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1. OBSERVATIONS ON THE USE OF NEMBUTAL AS A GENERAL ANÆSTHETIC IN THE PIG.
2. THE TECHNIQUE OF PARTIAL HEPATECTOMY IN THE PIG.
3. SPINAL (EPIDURAL) ANÆSTHESIA IN THE PIG.

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DURING the autumn of 1937 and the spring of 1938 a number of experiments were performed on pigs with the primary object of studying the pathogenesis of experimental cirrhosis of the liver.

In order to observe the toxic action of both single and repeated doses of carbon tetrachloride, and also to study the powers of regeneration of the liver, it was decided to subject the animals to a series of surgical operations under general anæsthesia with the object of removing portions of the liver for study. It was considered that it would, at the same time, afford a good opportunity to further our knowledge of the subject of anæsthesia in this species, and also serve to improve our surgical technique. In this communication the surgical aspect of the work only is referred to; the results of the pathological study will be published later (E. G. White).

Nembutal was the general anæsthetic employed. It was selected because of the consistently good results which have been obtained from its use in other species, particularly the dog, and also because it has not a toxic action on the liver itself which would tend to confuse the results obtained with the carbon tetrachloride.

The pigs used were of the large-white breed, and comprised two litters of 17 and 8 respectively, farrowed and reared in the Research Institute of the College. There being a number of males in addition to those used for experimental purposes, it was decided to examine the method of epidural anæsthesia for the operation of castration in these.

NEMBUTAL ANÆSTHESIA.

In the dog and cat it has been established that the most satisfactory method of administration of the drug is slow intravenous injection, assessing the depth of anæsthesia present as injection proceeds and stopping when the required depth is obtained. The injection should occupy a period of three to four minutes. In the healthy dog it is known that the quantity necessary to induce general anæsthesia of medium depth is between 24 and 35 mg./kg. (big animals taking rather less proportionately than small ones)*, and that anæsthesia will remain at its original depth for about 30 minutes, whilst the total duration of narcosis will be from four to eight hours. Very similar results have been obtained in the cat, horse and rabbit.

It was anticipated that large, fat pigs would require doses proportionately smaller than would small ones, and it was thought highly probable that animals which had previously been dosed with carbon tetrachloride would show an increased susceptibility to the anæsthetic.

In the dog the depth of anæsthesia is assessed upon three factors:—the onset of general muscular relaxation, complete relaxation of the lower jaw, and depression of the pedal reflex. In the pig it was thought that while assessment of depth would present no difficulties in small subjects, it might not be an easy matter in large ones.

In the pig the veins on the lateral aspect of the ear-flap are generally selected for intravenous injection, and in most cases this was the method adopted. In a number of very small subjects, however, the recurrent tarsal vein on the lateral aspect of the hock was used. In adults the thickness of the skin is such that it obscures this vessel. The adoption of one of the ear veins has disadvantages. They are small and penetration by the needle may be difficult. The amount of blood in them is slight and it may be difficult to draw blood into the syringe, a practice which is generally employed to ensure that the needle is in the vein. Unless the animal's head is very securely restrained during administration there is a tendency for it to be shaken, especially when some stimulus is being applied with a view to ascertaining the depth of narcosis. This generally results in the needle leaving the vein. Finally, since the vein is small the injury caused by the needle generally results in its subsequent thrombosis and fibrosis, so that it is not available for further injections at a later date. No difficulty was experienced in restraining animals up to 50 kg. The pig was placed upon a table either on its sternum or on one side, and while one assistant held the ear another applied pressure to the trunk. A similar method was adopted in

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* 47 mg./kg.—1/3rd grain per lb.
35 „ —1/4th „ „
29 „ —1/5th „ „
24 „ —1/6th „ „
20 „ —1/7th „ „

very large animals. This was, however, a mistake, for movement of the head frequently interfered with injection. In future the method recommended by Silbersiepe will be employed. A noose is passed around the upper jaw, behind the canine teeth, and fixed on either side to two immovable structures.

The pigs remained with the sow until they were eight weeks old, and after weaning their diet comprised a mixture of barley meal, maize meal and middlings, with a proportion of fish meal. They were not prepared for operation in any way. Suckling piglets were taken direct from the sow whilst older animals were given their last feed on the evening before operation.

Group 1.—This group comprised five pigs made up as follows:—

Litter	2.	Pig No.	4.	27 days old.	Weight	7·5 kg.
„	1.	„	24.	61 „	„	15·6 „
„	1.	„	17.	61 „	„	14·3 „
„	1.	„	5.	63 „	„	15 „
„	1.	„	18.	66 „	„	16 „

No. 17 was kept as a normal control and the remainder received carbon tetrachloride 0·6 c.c./kg. by subcutaneous injection two—three days prior to anæsthetization. In this group the animals were destroyed with a captive bolt pistol 10—15 minutes after the induction of anæsthesia. None of those which had received carbon tetrachloride showed any clinical signs of illness.

Pig No. 4. Nembutal in a 7 per cent. solution was slowly injected into an ear vein. At three minutes, when 0·2 grm. (28 mg./kg.) had been injected, general anæsthesia was present. Muscular relaxation was complete, the jaw was relaxed and there was no response to clipping of the skin with artery forceps. Injection was then continued until 0·3 grm. (40 mg./kg.) had been administered in four minutes. Anæsthesia was deep; respirations were slow and shallow. The heart beat was strong and the state was considered to be a safe one. An exploratory laparotomy was performed with a view to ascertaining the accessibility of the liver for operation and the animal was destroyed 15 minutes later while still in a state of deep anæsthesia.

This experience indicated that there was a considerable safety margin when inducing anæsthenia of medium depth in small pigs. It also showed that the dosage required was similar to that employed in the dog. A striking fact was that the previous administration of carbon tetrachloride did not apparently increase the animal's susceptibility, despite the subsequent histological finding that there was extensive destruction of the liver in this case.

Nos. 24, 17 and 18 were similarly given the solution by slow intravenous injection. Adminstration was continued until anæsthesia of medium depth was obtained. Care was taken that from 2½ to 3 minutes should be occupied during its induction. The dosage of nembutal was respectively 32, 31 and 34 mg./kg. Here again the previous administration of carbon tetrachloride did not increase susceptibility, for the control pig took rather less than the others.

No. 5 was given 0·5 grm. (33 mg./kg.) slowly into the recurrent tarsal vein. At four minutes there was no reaction to pinching the skin with artery forceps. A further 0·2 grm. was injected intra-peritoneally, making the total dose 46 mg./kg. Deep anæsthesia was present and an exploratory laparotomy was performed. A small piece of liver was snipped off with scissors. It was noticed that hæmorrhage was only slight.

Group 2.—Three pigs of litter 2.

No. 2. Female. Age 35 days. Weight 8·6 kg.
„ 1. Male. „ „ „ „ 9 „
„ 3. Male. „ „ „ „ 10 „

In this group it was proposed to remove, under nembutal anæsthesia, as much of one lobe of the liver as possible with a view to eliciting, at a later date, whether or not regeneration occurred. None of these pigs had received carbon tetrachloride.

Induction was effected by assessment and not by computation of a dose. No. 2 received 30 mg./kg., No. 1 28 mg./kg., and No. 3 25 mg./kg. Complete surgical anæsthesia for an interference of about 15 minutes' duration was obtained. No. 3 developed Cheyne-Stokes type of respiration during operation following rather severe hæmorrhage from the liver, but after a few artificial respirations together with carbon dioxide by inhalation breathing became regular again.

Three and a half hours later stimulation provoked shaking of the head and attempts to rise, while six and a half hours after operation the animals were able to walk. All fed vigorously the morning following the day of operation. At operation portions of liver varying from 11 per cent. to 20 per cent. of the whole organ were removed.

Group 3.—Two pigs of litter 2, Nos. 6 and 7.

First operation. No. 6. Female. Age 98 days. Weight 34·5 kg.
„ 7. „ „ „ „ 40 „

No. 6 had received previously six injections of carbon tetrachloride (0·2 c.c./kg.) at 5—6 day intervals. No. 7 was a normal control. The object of operation was the removal of a small portion of liver (about 5 grm.) for histological examination.

In each case anæsthesia was induced by assessment, No. 6 receiving 29 mg./kg. and No. 7 32 mg./kg. Anæsthesia was not quite deep enough and a little ether was given by inhalation during operation. Four hours later the animals were on the sternum and at six hours were able to walk. There was no post-anæsthetic malaise of any description.

Repeated doses of carbon tetrachloride appeared to have caused no increase in susceptibility and surprise was felt that the dosage required was slightly greater than for the smaller pigs.

Second operation. No. 6. Age 164 days. Weight 80 kg.
„ 7. „ „ „ „ 91 „

During the period elapsing between the first and second operations No. 6 received 13 injections (0·2 cc./kg.) carbon tetrachloride. The object of operation was again the removal of a small piece of liver for histological examination.

By this time the size and strength of the animals was becoming such that exact assessment of degrees of anæsthesia was difficult. Too vigorous stimulation during injection tended to provoke powerful head shaking, whilst relaxation of the abdominal muscles only was not an exact enough guide. The jaws were heavy and relaxation was difficult to assess. No. 6 was given a computed dose of 30 mg./kg. injection occupying 3½ minutes. It was thought that this would give medium depth anæsthesia. During preparation of the animal for operation movement occurred and a second injection was given increasing the dosage to 37 mg./kg. (Actual dose, 3 grm.) For this second injection a small vein on the inner aspect of the thigh was exposed by dissection. The control pig was given an initial dosage of 35 mg./kg. Good anæsthesia was present at the commencement of operation, but during suturing of the skin a little movement occurred and some chloroform by inhalation was administered.

Throughout these experiments the attitude adopted was that there should be absolutely no movement during surgical interference, and thus even when reflex movement occurred anæsthesia was deepened by inhalation. As far as our work had progressed it appeared that as pigs increased in size their susceptibility to nembutal was becoming diminished rather than increased.

Group 4.—Two male pigs already referred to in group 2.

No. 1. Age 100 days. Weight 40·5 kg.
„ 3. „ „ „ „ 32 „

The operation was that of castration.

Each animal was given 1 grm. of nembutal, representing a dosage of 25 and 31 mg./kg., by slow intravenous injection. Perfect anæsthesia for operation was obtained in each case. The so-called "uncovered operation" was performed, the spermatic cords being ligated with catgut. In one animal the scrotal wounds were left open and in the other catgut sutures were inserted in the skin. Recovery from the anæsthetic followed the usual course. No post-operative complications occurred and in each case healing was by primary intention.

Our experience in the dog has been that for superficial operations such as this the depth of anæsthesia necessary is less than that required for peritoneal interferences. This experience would tend to confirm that the same applies to the pig.

Group 5.—Three pigs, made up as follows:—

First Operation.

Litter 1.	No. 19.	Female.	Age 70 days.	Weight 29 kg.
„ „	„ 16.	„	„ 86 „	„ 35 „
„ „	„ 20.	„	„ 86 „	„ 36 „

At the time of the first operation none of these pigs had received carbon tetrachloride. Small pieces of liver (2—3 grm.) were to be removed surgically. The method of anæsthesiation employed was that of assessment of depth as injection proceeded. No. 19 received 0·8 grm. nembutal (26 mg./kg.) and Nos. 16 and 20 1 grm. (28 mg./kg.). Whilst anæsthesia was sufficiently deep for incision of the skin and abdominal wall, in each case a little movement of the limbs occurred during abdominal manipulation and so some ether by inhalation was administered. Five to six hours after operation the animals were up and able to walk, and all fed normally the following morning.

Seven days after operation, No. 19 was given carbon tetrachloride (0·2 c.c./kg.)—a dose which on previous experience appeared to be a perfectly safe one. The animal died 24 hours later. *Post-mortem* examination revealed extensive liver destruction. The operation wound had healed perfectly.

To this group was then added a normal pig of the same litter (No. 22).

SECOND OPERATION.

(First on No. 22.)

No. 16.	Age 123 days.	Weight 62 kg.
,, 20.	,, ,, ,,	,, 63 ,,
,, 22.	,, ,,	,, 59 ,,

No. 20 was the normal control while Nos. 16 and 22 had received repeated small injections of carbon tetrachloride. Again, small pieces of liver were to be removed for histological examination. A computed dose of 29 mg./kg. was administered to each. In all perfect anæsthesia was obtained which persisted throughout operation. In No. 20, consequent upon difficulty in restraining the animal, the injection was made rather more quickly than usual. Five minutes later respirations ceased, but the heart continued to beat forcibly. Pressure on the abdomen every 4 seconds provoked on each occasion a single, deep respiration. This was continued for about 10 minutes after which breathing became regular again. A striking feature of this apparent respiratory failure was that it was quite different from that seen in other species following bulbar paralysis, for expulsion of air from the chest was followed by a spontaneous, deep respiration, of the type seen when there is some mechanical obstruction to respiration during anæsthesia.

Three hours after operation, the three pigs raised their heads and grunted when stimulated, and from this point recovery was normal.

THIRD OPERATION.

(Second on No. 22.)

No. 22.	Age 222 days.	Weight 135 kg.
,, 20.	,, 229 ,,	,, 134 ,,
,, 16.	,, 239 ,,	,, 145 ,,

It will be observed that the repeated administration of carbon tetrachloride had not adversely affected increase of bodyweight in Nos. 16 and 22; in fact, reference to the chart on page 6 will indicate that despite the experimental interferences performed on these animals, their development was well above the average for the breed.

Nos. 20 and 22 were given a computed dose of 23 mg./kg. (3·2 grm.) injection occupying 2·25 minutes in the first and 4·5 minutes in the second. For convenience of administration the concentration of the solution was increased to 16 per cent. In both cases deep general anæsthesia developed and persisted throughout operations of 26 and 23 minutes duration. In both cases breathing ceased and it was necessary to apply abdominal compression. Here again pressure on the abdomen immediately provoked a deep, spontaneous inspiration. The belief that this cessation of breathing was due to mechanical factors rather than true respiratory failure was strengthened by the fact that in No. 22 raising of the head was immediately followed by return of regular respirations. It is quite possible that in a fat pig under deep anæsthesia that mere extension of the head is enough to cause pressure on the larynx sufficient to arrest breathing. Six hours after operation, No. 20 was walking about the sty apparently normal. In No. 22, recovery was delayed a little. At 8 hours it was up on its sternum but unable to walk. The following morning it was normal. Pig. No. 16 presented great anæsthetic difficulties. On the 236th day an attempt was made to anæsthetise it. Only part of the dose, however, was injected into the vein and much escaped into the perivascular connective tissue. Narcosis only was obtained. An attempt to complete anæsthetisation by the inhalation of chloroform provoked vigorous struggling and the operation was abandoned. Three days later a dose of 22 mg./kg. (3·2 grms.) was injected intra-peritoneally. Ten minutes' later, the pig became weak on its legs and lay down. At 15 minutes it was lying on its side in s state of narcosis, but muscular relaxation was incomplete and there was voluntary response to interference with the mouth. A further 1 grm. (increasing the dosage to 29 mg./kg.) was given into the peritoneal cavity. As this had no apparent deepening effect it was decided to continue by intravenous injection. A small vein on the inner aspect of the thigh was exposed by dissection and the needle introduced into it. 0·6 grm. was slowly injected and there was a deepening of narcosis. A further 0·8 grm. was given and light anæsthesia supervened (a sluggish pedal reflex). To ensure that there should be deep anæsthesia throughout operation a further 0·6 grm. was injected. The animal received a total dose of 6·2 grms. of nembutal (43 mg./kg.) of which 4·2 grms. was administered by intra-peritoneal injection and 2 grms. by intravenous injection. Five hours later, the animal grunted and attempted to rise when interfered with. The following morning it was normal.

Cases 20 and 22 indicate that when normal growth has ceased

and increase in bodyweight is due to fat deposition dosage, when computed according to bodyweight, must be reduced. No. 16 is difficult to understand. In the dog the effective intraperitoneal dose does not differ greatly from the intravenous one, and yet in this pig an intraperitoneal dose of nearly one-and-a-half times the intravenous one, provoked deep narcosis only. This leads to the suspicion that the injections were made into retroperitoneal fat rather than into the peritoneal cavity, an error it may be difficult to overcome in very fat animals. From this focus absorption is slow. At any rate, the case indicates that the liver of the pig is capable of dealing rapidly with large quantities of nembutal, for the duration of narcosis was only a little longer than in normal cases.

Throughout, the method of preparing the solution for injection was the same as that already described in detail by Wright and Oyler (*Vet. Rec.*, 1934, Vol. xiv, No. 49). The needle employed for intravenous injection was 1·2 c. long, 0·8 mm. outer diameter, with a " dental cut " point.

Discussion.

From the evidence afforded by these cases it would appear that nembutal administered by slow intravenous injection is a satisfactory general anæsthetic for the pig, and that for animals up to 90 kg., a dosage in the region of 29 mg./kg. injected in a period of three to four minutes will provoke anæsthesia of light to medium depth. In small pigs, there would appear to be a considerable safety margin, and since effective restraint is easy to apply in these the depth of narcosis and subsequently anæsthesia can be accurately assessed as injection proceeds. In larger subjects, restraint and assessment is difficult and it may be necessary to inject a computed dose. At the same time the onset of complete muscular relaxation is a fairly good guide to light anæsthesia, and to ensure safety it may be decided to stop when this point is reached and if necessary deepen temporarily by the inhalation of a little ether or chloroform. For very large animals (over 100 kg.) dosage must be reduced and to keep within safety limits not more than 20 mg./kg. should be injected. In the pig, deep general anæsthesia is always associated with a risk of respiratory failure. The experience using nembutal has been that provided the accident is observed early resuscitation is easy.

How might this knowledge be applied to veterinary surgery under conditions of practice ? The operation most frequently performed on pigs is that of castration. It is generally carried out when the animals are about 42 days old and weight about 10 kg. It must be admitted that when performed under local infiltration of the scrotum and spermatic cord the operation is painless although, perhaps, noisy. At the same time, the young pig is often the subject of scrotial hernia, when the performance of the radical operation is greatly facilitated by the induction of general anæsthesia. In some parts of the country, ovariectomy in young females is carried out.

Of this operation, the writer has had no experience but would suggest that the induction of general anæsthesia by the intravenous injection of nembutal would greatly facilitate operative technique. Sometimes the veterinarian is called upon to castrate larger pigs, animals weighing about 50 kg. In these local anæsthesia may leave much to be desired and it is suggested that either general or regional (epidural) anæsthesia would be an advantage. Finally, there are those operations sometimes carried out in the fully-grown, adult animal-castration of the boar, cæsarian hysterotomy, amputation of the prolapsed uterus, etc. For these general anæsthesia is essential and nembutal is recommended.

PARTIAL HEPATECTOMY—OPERATIVE TECHNIQUE.

Two, somewhat different, operations were performed. The first comprised the removal of comparatively large portions of liver, from 11 to 20 per cent. of the whole organ; and the second the removal of small pieces, from 2 to 5 grms. only.

Having induced general anæsthesia the animal was placed upon a table in the dorsal position with the limbs extended and held in this position by tapes. The ventral aspect of the abdomen and chest was thoroughly washed with hot water and soap, shaved, dried, and finally washed again with 90 per cent. alcohol. The whole of the animal, with the exception of the aperture through which operation was to be performed, was covered by calico cloths held in position by towel-clips. For swabbing, gauze was employed in some cases and absorbent paper in others. Cloths and swabs were sterilised by autoclave. Instruments were boiled in tap water for 10 minutes, and after removal from the steriliser placed on trays covered by sterile battiste cloths. Rubber gloves, sterilised by boiling, were worn. Throughout, all buried ligatures and sutures were of catgut sterilised by the makers (Johnson and Johnson). In the early cases, silk was used for suturing the skin, but later catgut was employed throughout. This overcame the necessity for subsequent removal of the skin sutures. On completion of the operation the wound was covered with several layers of sterile gauze (or absorbent paper) held in position and completely covered by 3-inch adhesive tape. To give support to the abdomen and to ensure that the strapping did not become detached, the tape was passed around the body.

Operation 1.—Was performed on three pigs 35 days old and weighing from 8·6 to 10 kg. (Two of these were further subjected to the operation of castration when 100 days old and weighing 32 and 40·5 kg. respectively.)

Pig No. 2.—An incision 7 cm. long was made parallel with and, 2 cms. behind the costal arch on the right side, through the skin, abdominal muscles, fat and peritoneum. Bleeding was not severe. One arterial point—the anterior abdominal, was picked up with Spencer-Wells forceps and ligated. The ventral border of the right lateral lobe of the liver lay immediately beneath the incision. The

lobe was drawn out of the abdomen as far as possible. An encircling gut ligature was placed high up, around the base of the lobe and tightened until it was seen to be commencing to cut through the edge of the lobe. The portion of the liver distal to the ligature was then cut off with scissors along a line about 1 cm. distant from the ligature. Hæmorrhage, which comprised a little oozing from the cut surface, was not serious and no further attempt to check it was made. The abdominal wall was closed by two layers of interrupted sutures. The first, comprising three sutures, included the peritoneum and abdominal muscles, and the second, four sutures, the skin.

The weight of liver removed was 55 grms. and it was estimated that this represented 14 per cent. of the whole organ. Recovery was uneventful, and healing by primary intention occurred.

Eleven days after operation, this animal was destroyed. *Post-mortem* examination revealed that the greater part of the right lateral lobe of the liver had been removed. There were firm adhesions of the peritoneum and diaphragm to the stump of the lobe. No evidence of regeneration, either locally or diffusely throughout the organ, was observed. It is probable that insufficient liver tissue had been removed.

Pig. No. 1.—The technique of operation was the same as for the previous case. It was possible to apply the ligature higher up, in fact, it was insinuated in the fissure between the right lateral and caudate lobes ; 70 grms. (20 per cent. of the whole organ) was removed. Hæmorrhage from the cut surface was more severe than in the previous case. Recovery from both the anæsthetic and the operation were quite uneventful, and healing was by primary intention. This animal was destroyed when 144 days old, 109 days after operation. At *post-mortem* examination, the site of the original operation could not be found. There was no right lateral lobe of the liver. The liver was the heaviest of any animal examined at any age. It weighed 2,650 grms. and had undergone a diffuse compensatory hypertrophy.

Pig. No. 3..—Technique as before. In this case, the right central lobe was attacked. Difficulty was experienced in introducing the ligature sufficiently high up to snare the lobe of the liver. It was, in fact, quite ineffective. Hæmorrhage was most severe and it was thought that it would prove fatal. To our surprise recovery from anæsthetic and operation was as usual. When 129 days old, 94 days after operation, this animal was given carbon tetrachloride (0·6 c.c./kg.) by subcutaneous injection. This caused no apparent ill-health. Destruction was carried out six days later. At *post-mortem* examination no operation scar could be found. The greater part of the right central lobe of the liver had been removed together with most of the gall bladder. This was thought to be a congenital bile duct cyst at the first operation because it was embedded in the lobe of the liver. There was very little scar tissue and no evidence of regeneration.

Operation 2.—This was performed on 13 occasions, in animals varying in age from 70 to 239 days, and in weight from 29 to 145 kg., made up as follows :—

One animal—on one occasion only.
Three animals—on two occasions.
Two animals—on three occasions.

From the viewpoint of operative technique, the cases fall into three groups according to bodyweight. The first—weights from 29 to 40 kg., comprised Pigs 19, 16, 20, 6 and 7.

A mid-line skin incision 7 cm. long was made, its anterior commissure being immediately posterior to the xiphisternum. The incision, reduced in length to 5 cm. was continued through the abdominal muscle and peritoneum parallel with but about 1 cm. to the right of the linea alba. The ventral border of the right central lobe of the liver appeared in the incision. It was drawn out of the abdomen and lightly gripped with bowel forceps (the jaws of which were protected by rubber tubing). In No. 19, a small piece of liver (about 2 grms.) was snipped from the ventral border with scissors. Relaxation of the forceps indicated that hæmorrhage was negligible and the lobe was allowed to recede into the abdomen again. In the remaining cases of this group the border of the lobe was ligated, above the line of section by means of two, transfixing, gut ligatures. This practice was adopted in all future cases, and it served completely to check hæmorrhage from the cut surface. It was found that in older pigs, due to the greater amount of inter-lobullar connective tissue, much greater tension could be applied to the ligatures without a risk of tearing the liver.

The abdomen was closed by two layers of interrupted sutures, three in each. The first included the peritoneum and abdominal muscle, and the second the subcutaneous fat and skin. The wound was covered with a sterile pad and adhesive strapping was applied. The time of operation, from the commencement of the skin incision to completion of the suturing, was 17 minutes in the first case. This time was reduced in subsequent operations until in the last it was only 7 minutes 40 seconds.

The second group was composed of animals weighing from 60 to 90 kg. (With the exception of No. 22, it was the second operation on the pigs included in the first group.) The technique was almost the same as that previously described. The incision was made parallel with but a little to one side of the scar of the previous operation. The greater thickness of the abdominal wall necessitated an increase in its length to 8 to 9 cm. A small piece of liver was removed either from the margin of the right central lobe a little to one side of the previous scar, or from the ventral border of the left central lobe. Exposure of the lobe was facilitated by seizing it with vulsellum forceps and gently withdrawing it from the abdomen. Suturing of the abdominal wall was a little slower because the scar of the first operation tended to mask the respective layers. The time occupied

by operation in those cases in which it was not interrupted by the application of artificial respiration was 11 to 12 minutes.

In the third group the pigs weighed from 134 to 145 kg. In two animals it was the third operation and, in the remaining one, the second. The technique of operation was essentially the same as that described for group two, but both incision and closure of the abdomen was a much more cumbersome task. The average time of operation was 25 minutes.

In every case healing was by primary intention and there was no post-operative malaise of any kind. The morning following operation the animals took their food normally, and their progressive increase in bodyweight was affected only slightly if at all.

Discussion.

This work indicates that the pig is a good subject for experimental surgical interferences with the liver. Provided that relative asepsis is observed it is reasonable to expect healing by primary intention in every case. The animals appear to suffer no shock as the result of operation and after recovery from the anæsthetic act as though no interference had been made. From the aspect of facility in handling and induction of anæsthesia, pigs up to 50 kg. bodyweight are most suitable. Over this weight their size, strength and general unwieldiness present difficulties.

EPIDURAL ANAESTHESIA.

The application of epidural anæsthesia in surgery of the pig has received some attention on the Continent, but, as far as the writer knows, has not been exploited in this country.

In the pig, the spinal cord ends at the junction of the fifth and sixth lumbar vertebræ, and the spinal meninges continue, around the phylum terminale, as far as the middle of the sacrum. At the lumbo-sacral space the sac is comparatively small and it is improbable that a needle introduced at this point will penetrate it and that the injection will thus be made into the sub-arachnoid space. The limbo-sacral aperture is large, its dimensions in an adult being approximately 1·5 cm. in the antero-posterior direction, and 3 cm. transversely. The depth of the canal is about 1 cm.

The site for insertion of the needle is located as follows: The anterior border of the ilium on each side is found with the fingers. A line joining them crosses the spinous process of the last lumbar segment. The needle is inserted in the mid-line, immediately behind this spine, and directed downwards and backwards at an angle of about 20° with the vertical. The depth to which the needle must penetrate in pigs of from 30 to 70 kg., will vary from 5 to 9 cm. Whilst the landmarks outlined may be readily detected in animals of smaller size they may be entirely masked by the overlying tissues in larger ones. In these, a point 15 cm. in front of the base

of the tail serves as a fairly accurate guide. Provided the needle is introduced in approximately the correct position the size of the lumbo-sacral aperture makes its detection comparatively easy. The needles used were of the type introduced for bovine epidural injection, 1·5 mm. outer diameter. For pigs between 30 and 50 kg. one 8 cm. long was employed. In one animal of 70 kg. a 12 cm. needle was used.

Before inserting the needle an insensitive skin weal was provoked by injecting local anæsthetic into the dermis at the appropriate spot using an ordinary, short hypodermic needle. The animal was restrained either on its breast or on its side. The latter was the better method for by it sudden movement was more effectively controlled. In all cases penetration of the canal by the needle was associated with sudden movement on the part of the animal. (Similar movement generally occurs in the cow). The anæsthetic solution was warmed before injection by placing the sealed ampoule containing it in recently boiled water. The syringes employed were kept immersed in alcohol. Immediately before use they were rinsed in boiled water. This served not only to remove the alcohol, but also to warm them. Needles were sterilised by boiling. Bristles were clipped from the animal's back over an appropriate area and the site cleansed with alcohol. Injection was made comparatively quickly, occupying from 5 to 10 seconds.

Case records :—

1. *Male. Age*—100 *days. Weight*—32 *kg.*

5 c.c. of a 2 per cent. tutocain solution with 0·0005 per cent. suprarenin (Bayer) was injected into the epidural space. Injection was made with the animal on its breast, and it was kept in this position throughout. Five minutes later the tail was completely relaxed but the scrotum was still sensitive. The operation of castration was performed under general (intravenous nembutal) anæsthesia.

2. *Male. Age*—105 *days. Weight*—40 *kg.*

10 c.c. of the 2 per cent. tutocain-suprarenin solution was injected. The animal was restrained on its right side during injection and kept in this position throughout. Tail relaxation was almost immediate. At five minutes clipping of the scrotum over the lower testicle with Spencer-Wells forceps provoked no response. At ten minutes the operation of castration was commenced. The lower testicle was removed under perfect anæsthesia. Incision of the scrotum over the upper testicle provoked a jerk in the hind limbs, although during completion of the operation, including suturing of the skin, there was no movement. There was partial motor paralysis of the hind limbs. The animal was able to move them vigorously but was unable to raise its hind quarters from the ground. Two hours later the pig was moving normally and its tail was curled.

3. *Male. Age*—120 *days. Weight*—49 *kg.*

10 c.c. of the 2 per cent. tutocain-suprarenin solution was injected. The animal was restrained on its breast and in view of the unequal anæsthesia which was obtained in case 2 when the animal was in the lateral position, it was kept on its breast throughout. At one minute the

tail was relaxed. At eight minutes the scrotum on each side was slipped with forceps without response. At nine minutes the operation of castration was commenced. It was completed under perfect anæsthesia. Motor interference with the hind limbs was a little greater than in case 2. Two hours later the animal was up and walking about.

In none of these cases were there any observable after-effects.

4. *Castrated Male. Age*—144 *days. Weight*—70 *kg.*

In this case a 12 cm. needle was employed, the depth of penetration necessary being 9 cm. 20 c.c. of the 2 per cent. tutocain-suprarenin solution was injected. During injection the animal was restrained on its side, but on completion it was allowed to rise and no further restraint was applied. At one minute the tail was limp and hind limb disability was evident. At five minutes skin anæsthesia as far forward as the anterior region of the sternum was present. The hind limbs were completely paralysed and the abdominal muscles were completely relaxed. There was a partial paralysis of the fore limbs. Respirations appeared to be entirely diaphragmatic although there was no distress, nor was there any evidence of shock.

At twelve minutes the animal was destroyed, using a Temple-Cox captive bolt pistol.

From the scanty evidence afforded by these cases it is impossible to draw any definite conclusions. At the same time the results indicate that for the castration of pigs of from 30 to 50 kg. the method of epidural anæsthesia is worthy of further trial.

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Acknowledgements.—This work was performed in collaboration with Mr. E. G. White, of the department of Pathology of the College, who is carrying out the pathological study. I am indebted to him for the help he gave me during the operations and also for keeping records.

180
160
140
120
100
BODY
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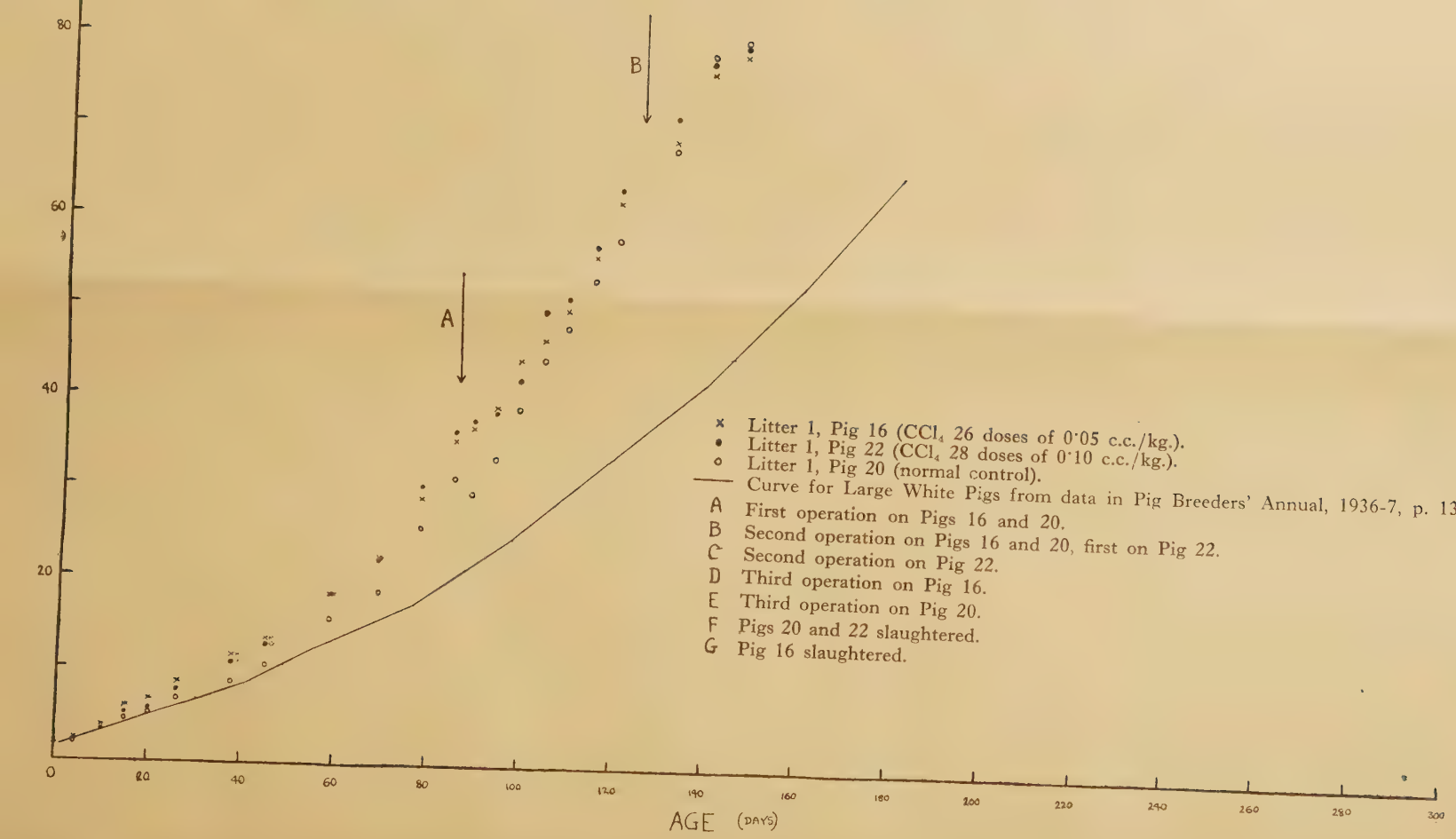
F


80
60
40
20
0
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200
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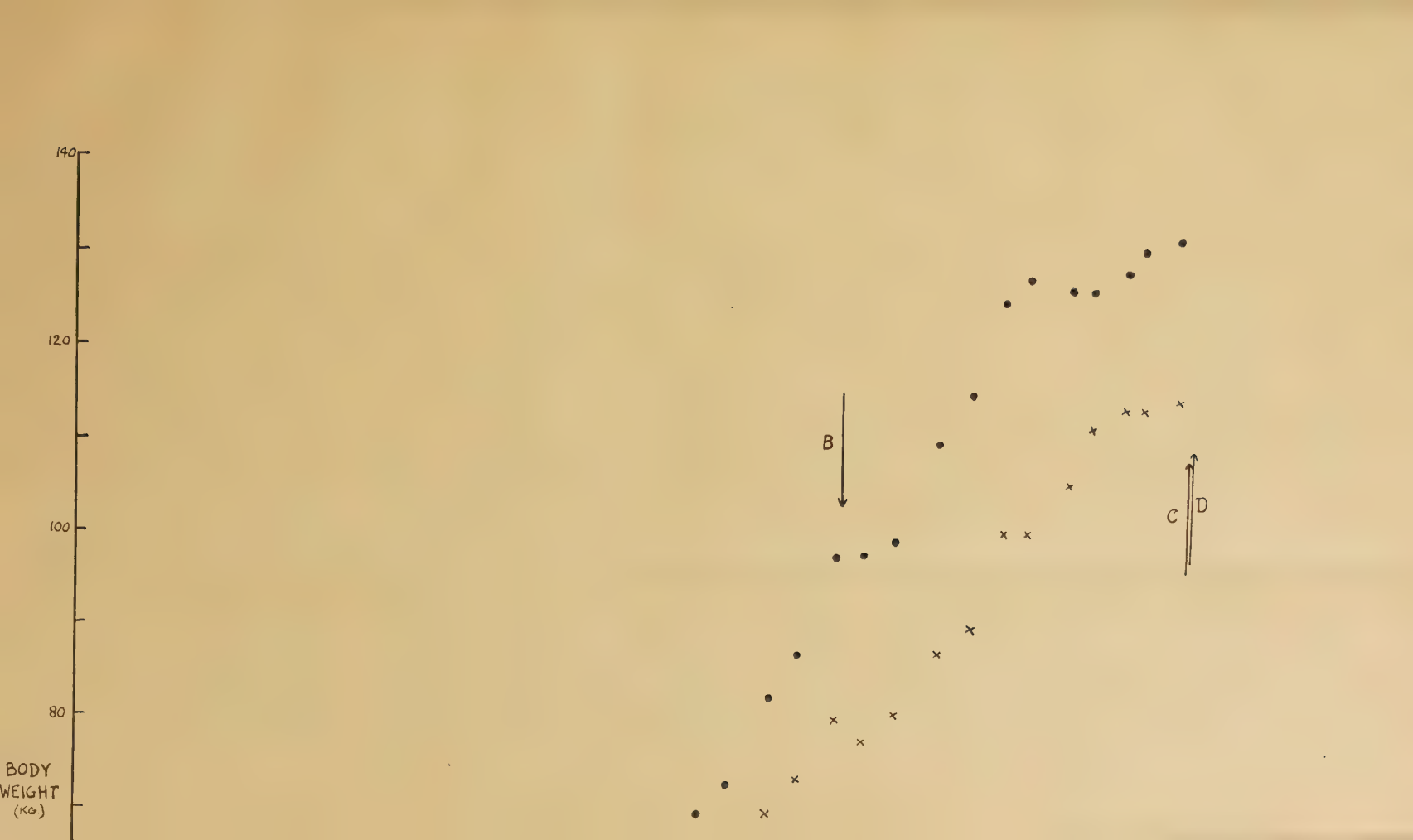
300
AGE (DAYS)
A
B
× Litter 1, Pig 16 (CCl_4 26 doses of 0·05 c.c./kg.).
• Litter 1, Pig 22 (CCl_4 28 doses of 0·10 c.c./kg.).
o Litter 1, Pig 20 (normal control).
— Curve for Large White Pigs from data in Pig Breeders' Annual, 1936-7, p. 132.
A First operation on Pigs 16 and 20.
B Second operation on Pigs 16 and 20, first on Pig 22.
C Second operation on Pig 22.
D Third operation on Pig 16.
E Third operation on Pig 20.
F Pigs 20 and 22 slaughtered.
G Pig 16 slaughtered.


140
120
100
80
BODY WEIGHT (KG.)
B
C
D

EXPERIMENTS ON THE TRANSMISSION OF BOVINE CONTAGIOUS PLEURO-PNEUMONIA WITH A REPORT ON A NEW METHOD OF TESTING IMMUNITY FOLLOWING VACCINATION

By R. W. M. Mettam and J. Ford,

Veterinary Laboratory, Vom, Nigeria.

The impossibility of producing lung lesions under experimental conditions has long handicapped the investigation not only of the pathology of the disease but also the exact mode of transmission. No matter what the route of inoculation chosen or the nature of the inoculum, *i.e.*, virulent culture or material from an acute case of the disease, the results were consistently negative.

The most that could be done was to produce by intrathoracic inoculation a hydrothorax similar to that frequently found in natural cases, sometimes complicated by peritonitis, serous inflammation of lungs and acute inflammation of the mediastinal glands.

Subcutaneous swellings can be produced by culture or lymph. These are of an œdematous nature and on histological examination resemble the specific lung lesion of the natural disease. Intravenous inoculations are invariably unsuccessful provided that the perivascular or subcutaneous tissues are not contaminated by the inoculum. Theiler claimed to have set up a reaction and subsequent immunity by drenching with cultures or lymph but Nocard and others have been unable to confirm these results.

Attempts to transmit the disease through the air passages have only succeeded twice, once by Chaveau and Nocard in their classical experiment and again more recently by Daubney (1935).

Contact experiments in the field are not at all satisfactory and an example is afforded by an experience of Walker (1929), who exposed susceptible cattle in highly infected herds for periods up to seven months. Only 41 per cent. of the in-contacts contracted the disease, though the remaining 59 per cent. were subsequently shown to be still susceptible.

To Daubney (1935) belongs the credit for producing in several animals characteristic lung lesions of the disease. He adopted the method devised by Tuttle (1933) for the production of experimental pulmonary abscesses in the dog. By the intravenous injection of agar emboli containing lymph or the recently isolated organism he produced characteristic lesions of bovine-pleuropneumonia. These results were quickly confirmed in Australia by Turner, Campbell and Dick (1935). Since then no further publications on this very interesting subject have appeared.

Daubney also pointed out the possibilities of the embolus method as a means of testing immunity following vaccination, though he himself did not undertake any experiments to prove the prophylactic power of the Kabete vaccine.

The primary object of our experiments performed in Nigeria was to confirm Daubney's results and, if satisfactory, to carry out an immunity test on vaccinated cattle.

A reliable laboratory method to test immunity conferred by culture vaccine has long been needed.

Hitherto, the usual procedure for testing the resistance of vaccinated animals was either to place them in close contact with active cases of the disease or to inoculate them with virus, subcutaneously behind the shoulder, not earlier than six weeks after tail vaccination.

Both methods have serious defects. For instance, the difficulty of producing contact infection even under experimental conditions has already been mentioned, and Walker's experience in Kenya may be described as a typical one. Further, the result of inoculating a dose of lymph or virulent culture into the subcutaneous tissues behind the shoulder to test immunity following previous tail vaccination is often difficult of interpretation, since some animals considered successfully tail-vaccinated (the criterion being a local reaction) will on subsequent shoulder inoculation give a swelling of varying size which ultimately subsides. In our experience, a large percentage of susceptible animals will similarly react to shoulder inoculation.

The embolus method by its ability to reproduce the classical disease has, as will be described in this paper, been proved to be the most practical method of testing vaccine immunity.

For the experiments to confirm Daubney's results, 17 animals were used. They were obtained locally but their histories were not known with certainty, though they came from a part of the country in which vaccination against pleuro-pneumonia had not been carried out for some time, and in which the disease has not appeared for years. It was expected, therefore, that the majority would be susceptible. It may be explained that approximately 40 per cent. of adult native cattle in this country, in which the disease is enzootic, are resistant, so that in order to obtain clear cut results it is necessary to put a large number of animals under experiment. As will be seen later in this paper, out of 17 so called susceptible animals used by us, six, that is one-third, proved to be resistant.

In addition to the 17 which were inoculated intrajugularly, one animal was injected with an agar plug plus culture into the lung parenchyma and two others received similar material direct into the pleural cavity.

For the vaccine immunity test, ten animals were obtained from the departmental Stock Farm and all were known to have been vaccinated at least once.

Before describing the experiments in greater detail, a brief account will be given of the technique employed.

Technique.

The emboli were prepared by mixing 5 per cent. virus or culture with 10 per cent. agar cooled to 40° C. The agar mixture was then aspirated into cannulæ, 90 mm. long with a bore of 9 mm. Several emboli measured 80 mm. by 8 mm. and formed a long, fine, well-set plug. They were thus of substantial size, and effectively occluded comparatively large vessels. As will be seen later, they persisted more or less intact in the veins for several months, and could be easily felt in the parenchyma during manipulation of the lung. It may be mentioned that in no case were any immediate symptoms of respiratory distress noted as a result of inoculation of these emboli.

In order to avoid any local reaction due to escape of virus into the perivascular and subcutaneous tissues, great care was taken to inoculate the emboli directly into the blood stream. A cannula of slightly larger bore than the one containing the agar was used to puncture the vein. The second and smaller cannula was thrust into the first, and the agar driven into the vein by the trocar, whose sharp tip had been blunted to prevent smashing the embolus. After withdrawing the smaller cannula, about 50 c.c. saline were injected in order to wash off any traces of agar that might be adherent to the larger cannula, and thus reduce the risk of soiling the tissues during removal. As a further precaution pressure was applied to the vein so as to cause blood to flow through the cannula prior to withdrawal. When the large cannula was pulled out the wound was allowed to bleed freely for a minute or so, as a final flushing of the skin and subcutaneous tissues. These precautions may appear excessive, but they successfully prevented the escape of virus into the connective tissues. In the thirty animals inoculated by us, not a single one showed any local reaction, whereas previous workers have reported skin infection with pleuro-pneumonia virus or culture resulting in serious local lesions.

Experiment No. 1.

Ten locally purchased cattle were inoculated intravenously on May 4th, 1938, by the technique described above, shoulder lymph being used to impregnate the emboli. The reactions and *post-mortem* histories of the animals, all of which were destroyed for experimental observations, will now be described in some detail.

Bull No. 1,711.—The animal was slaughtered 77 days after inoculation. At no period were any symptoms noticed. There was a circular area, reddish in colour, of a fibrinous nature, about the size of a walnut, on the parietal pleura covering the upper third of the right seventh rib. In the right lung, corresponding in position to the pleural lesion, was a hard circumscribed area, 4½ cm. by 2 cm. lying immediately under the visceral pleura. On section this was found to be a typical sequestrum with a poorly developed capsule.

Bull No. 1,800.—The animal was slaughtered 74 days after inoculation. No clinical symptoms were reported at any time. At

autopsy both lungs showed extensive lesions. There were seven in the left lung and twenty in the right. They varied in length from 0·75 cm. to 5 cm. All were sub-pleural in position, had a homogeneous greyish pink colour, and were firm in consistence. All showed evidence of characteristic marbling, though there was no sequestration and no encapsulation. The largest measured 5 cm. by 2 cm. The overlying pleura was thickened and pearly white in colour. The costal pleura was unchanged.

Bull No. 1,803.—This animal was destroyed 81 days after inoculation. There was a slight rise of temperature from the 22nd to the 27th day, but no clinical symptoms were recorded. At *post-mortem* a few fibrinous adhesions were found connecting the main lobes to the diaphragm. Both lungs showed recently cicatrised areas, whitish in colour, around which was much puckering of the neighbouring lung tissues. Soft pinkish fibrinous adhesions were noted along the free convex borders of both lungs, forming a lace-like festoon.

Bull No. 1,427.—This animal was killed 80 days after inoculation. It showed a sharp thermal reaction from the 8th to the 14th day. No clinical symptoms were reported. On opening the thorax, scattered, soft, fibrinous deposits of recent origin were seen adherent to the pulmonary pleura. They were most numerous over the costal surface of the main lobes. During manipulation of the right lung, a firm body, 0·75 cm. in length, was felt in the depth of the costo-vertebral border just posterior to the hilus. On section the nodule was found to be a part of a well-preserved agar embolus plugging a small blood vessel. Adjacent to this thrombosed vessel was a small hepatised area, brownish in colour, in which there was a distinct area of mosaic or marbling in the strands of which were beads of lymph. The area was oval in shape and 1 cm. in length. Cicatrised lesions were observed in the cardiac lobe of the left lung, causing contraction of the adjacent lung tissue. Over this scar the pleura was thickened and opaque. Both lungs were unusually voluminous and doughy on manipulation.

Bull No. 1,799.—After being kept under observation for 78 days, the animal was destroyed for detailed examination. There was no thermal or clinical reaction. At *post-mortem* extensive adhesion was seen over an area measuring 12·5 cm. by 30 cm. between the base of the right lung and diaphragm. The left lung contained two characteristic sequestra, each the size of a haricot bean, sub-pleural in position and lying in the convex free edge of the main lobe towards the posterior extremity. Capsules were well developed. The right lung also contained lesions. Two sequestra similar to those noted in the left lung were found lying deep in the free border of the main lobe. Over all the sequestra the pleura was much thickened and concealed by soft, pinkish deposit. The cardiac and anterior lobes were connected by similar material. Over the anterior half of the anterior lobe of the right lung was a pale yellow omelette-like mass,

firmly adherent to the underlying pulmonary pleura, but free from the parietal pleura. There were no lung lesions under the deposit. Both lungs were more œdematous than normal.

No lesions were found in the five remaining cattle.

Remarks.

The result of this experiment can be regarded as highly satisfactory, since pronounced lesions of chronic contagious pleuro-pneumonia were obtained in five out of ten animals. Further, they amply confirm Daubney's opinion that the embolus method is of great value in the experimental study of the disease.

However, it was decided to initiate a second experiment in order to extend the observation and, if necessary, to destroy the animals at a much earlier period after inoculation so as to obtain pathological lesions at a more acute stage.

A detailed discussion on these experiments will be included in the general remarks at the end of the paper.

Experiment No. 2.

No thoracic or " tumour " lymph was available at the time this experiment was planned, so it was necessary to use a fifth generation subculture in the emboli. The same technique was adopted and again no local reactions were observed.

The animals were all local purchases, but were obtained from a different source to those used in the previous experiment.

Seven of them were inoculated intravenously with the agar emboli. In addition, in two animals the inoculations were made intrathoracically, while in one an embolus was injected deep into the left lung.

All animals were destroyed between 22 and 28 days after inoculation. Details of the cases will now be given at greater length.

(*a*) *Intrapulmonary inoculation.*—Ox No. 2,072 was inoculated with an embolus into the left lung. The period of incubation was five days, but the temperature did not reach its maximum of 103·8° until the 8th day after inoculation. Subsequently it fell gradually, but showed slight fluctuations until the animal was destroyed on the 24th day. Coughing was first noted 14 days after inoculation and continued until death.

The animal lost condition very rapidly. On *post-mortem* examination, very marked extensive pleuro-pneumonia lesions were found involving the whole of the left costal pleura which was covered by a pinkish gelatino-fibrinous deposit averaging 1 cm. in thickness. (See Fig. 1.)

A similar deposit existed on the left half of the diaphragm and there was a moderate hydrothorax. While removing the left lung from the chest wall, three sequestra were cut through, the largest of which measured 4 by 2·5 cm. with a capsule 0·4 cm. thick. (See Fig. 2.)

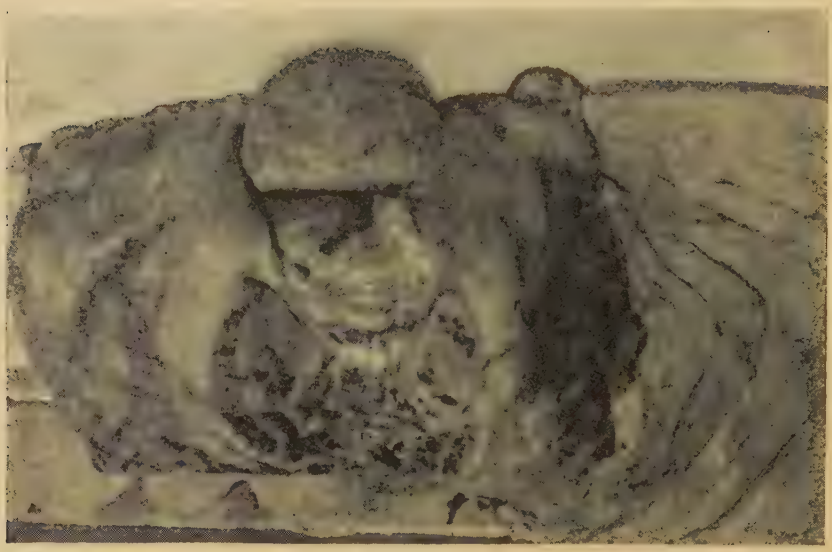


FIG. 2.

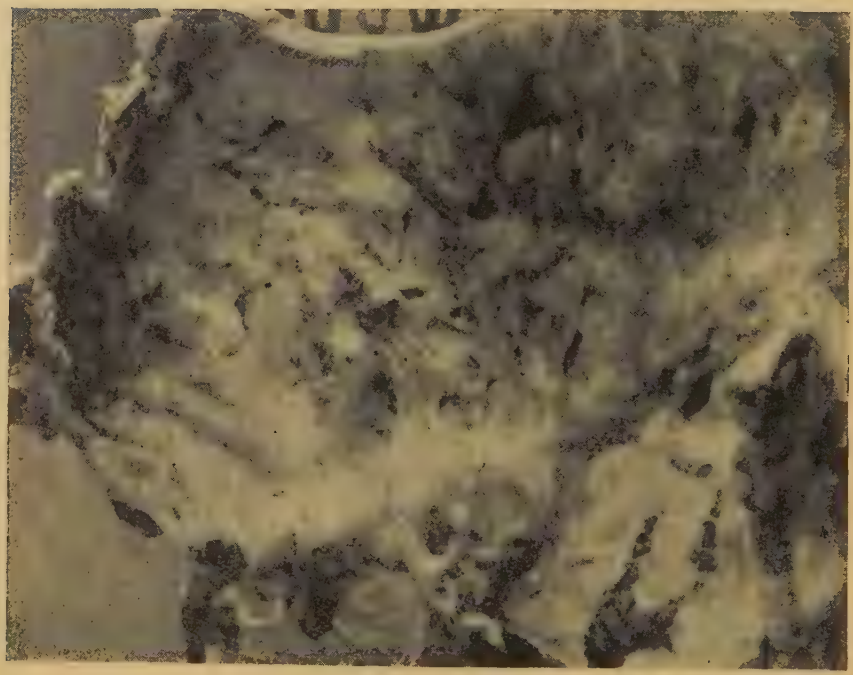


FIG. 1.

The smallest sequestrum was the size of a pea. Deep to the sequestrum in the main lobe was a patch of characteristic pleuro-pneumonia tissue measuring 12 by 9 cm. which showed typical marbling. (See Fig. 3.)

The rest of the lung was œdematous. The bronchial glands were much hypertrophied, granular and very juicy. The right lung was normal.

FIG. 3

(*b*) *Intrathoracic inoculations.*—In these two cases the agar embolus was introduced into the right pleural cavity. In Ox No. 2,074 there was a slight rise of temperature six days later which persisted for seven days. Coughing was first reported on the 19th day after inoculation. The animal was destroyed on the 24th day. On *post-mortem*, there was an extensive fibrinous pleurisy attaching the main lobe of the right lung to the diaphragm and opposing chest wall. The parenchyma of both lungs was free from lesions.

In Ox No. 2,076, the temperature rose on the 8th day to 103° F. and persisted for ten days. The clinical symptoms recorded were coughing, nasal discharge, grinding of teeth and gradual loss of condition. The animal was destroyed on the 24th day. At *post-mortem* the lesions were similar to those of No. 2,074 (*vide supra*).

(*c*) *Intrajugular inoculations.*—Ox No. 2,070. Its temperature rose on the 9th day and persisted for one week. The chief symptom was frequent and painful coughing, first reported on the 14th day and persisting till death. There was also a gradual loss of condition. The animal was destroyed on the 26th day after inoculation. The pleura was perfectly healthy. The right lung showed six sequestra all situated in the posterior half of the right main lobe. They varied in size from an almond to a golf ball. Encapsulation was complete and surrounding each lesion was an area still showing marbling and red hepatisation. The bronchial glands were enlarged and juicy.

Ox No. 2,071. After an incubation period of seven days, there was a marked thermal reaction, which persisted throughout life. Coughing was first noticed on the 15th day and remained until death. The animal was destroyed on the 26th day after inoculation. At *post-mortem* there was a marked bilateral hydrothorax. Both main lobes were firmly attached to the diaphragm and adjacent part of the chest wall. In the posterior portion of the right main lobe were two lesions, one the size of a haricot bean and the other measured 4·5 cm. by 3 cm., the latter being surrounded by a dense capsule 1·5 cm. thick. On section the large lesion was found to envelop about 15 small sequestra. The overlying pulmonary pleura was much thickened and pearly white in colour. In the main lobe of the left lung were seven sequestra, the largest of which was the size of a golf ball. All were encapsulated and surrounded by a zone of typical euro-pneumonia marbling.

Ox No. 2,073. The period of incubation was five days and was followed by a very sharp thermal reaction lasting for ten days, the fastigium being 105° F. Clinical symptoms included coughing, gradual loss of condition and increasing apathy. The animal was destroyed on the 25th day after inoculation. On *post-mortem* examination a moderate hydrothorax was observed. There were no lesions on the costal pleuræ. The right lung was much more voluminous than normal, and a large semi-coagulated mass, yellowish in colour covered the lower half of the right main lobe, the cardiac lobe and extended on to and covered the pericardium. The deposit in places was nearly 0·75 cm. thick. The right main lobe was firmly adherent to the opposing surface of the diaphragm. Within the parenchyma of the main lobe towards its posterior extremity, underneath the pleural deposit, was a large firm lesion measuring 9 by 7·5 by 3·75 cm. (See Fig. 4.)

On section it proved to be a typical pleuro-pneumonia sequestrum surrounding which was a zone of characteristic marbling.

Some of the veins of the marbling were very large, measuring more

than 0·2 cm. in width and contained lymph. The left lung was normal.

Ox No. 2,075. Period of incubation was seven days. There was a very noticeable thermal reaction, which remained until the animal was destroyed on the 22nd day. Coughing was first recorded on the 7th day and persisted throughout life, becoming more frequent and even paroxysmal. On the 14th day respirations reached 30 per

Fig. 4.

minute, and maintained this rate for the rest of life. The ribs became fixed and the costal arch stood out prominently. Loss of condition was very rapid, the animal becoming dull and depressed. On *post-mortem* examination both lungs and pleuræ were affected. On the left side, adhesions connected the distal thirds of the 5th and 6th ribs to the opposing lung by whitish strands forming a rope-like mass measuring 5 cm. by 3 cm. On the right side, the lower border of the right cardiac lobe was firmly fixed to the opposing chest

wall over the 5th and sixth inter-chondral spaces and the adjacent edge of the sternal origin of the diaphragm. This mass of adhesion measured 4 cm. in width and 2 cm. in length. Besides these lesions the costal pleura showed small scattered sero-fibrinous deposits. The pulmonary pleuræ were generally thickened and opaque, very noticeably so over the sequestra to be described later. In places both lungs showed a peculiar shrivelled appearance, though both organs were more œdematous than normal. There were three lesions in the left lung and eight in the right. Of these, ten were situated in the posterior part of the bases, the remaining one lying in the depth of the right costo-vertebral border behind the hilus, near one of the large bronchi. In the centre of this isolated lesion were found the remains of a readily recognisable agar embolus. All lesions were characteristic pleuro-pneumonia sequestra. External to the capsules were areas of typical marbling, in the mosaic of which could be seen large beads of lymph. The largest lesion measured 8 by 6 cm. and was in the base of the left lung.

Ox No. 2,077. The incubation period lasted seven days. Thermal reaction was mild but distinct, and persisted throughout life. Coughing was first recorded on the 11th day. Other clinical symptoms included persistent shivering and gradual loss of condition. The animal was destroyed on the 25th day after inoculation. At *post-mortem* examination there was a moderate hydrothorax. The costal pleuræ were healthy. The right lung was more voluminous and œdematous than normal. There were four typical sequestra, all situated towards the posterior extremity of the right main lobe, the largest being the size of a golf ball. All were surrounded by areas showing characteristic marbling.

Ox No. 2,078. Period of incubation was eight days. There was a very marked thermal reaction, the temperature rising daily to a peak of 106° F. on the 17th day, after which it dropped gradually to 101° F. on the 24th day, when it again rose to 104° F. Coughing was recorded on the 10th day and became more and more exacerbated as the disease progressed. Respirations became accelerated and on the 17th day were counted at 44 per minute. They maintained this rate until the animal was destroyed. There was also a muco-purulent nasal discharge. The ribs stood out prominently and were fixed, and the respirations became shallow, painful and abdominal. The animal lost condition very rapidly and was easily distressed. It was slaughtered on the 24th day after inoculation, the 16th day of illness. On *post-mortem* examination the costal pleuræ were found to be quite healthy. There was no hydrothorax. Both lungs were much more voluminous than normal, and showed very numerous reddish brown hepatised areas involving one or more lobules, very firm in consistence and somewhat granular in appearance. The lesions were of a more acute nature than the previous cases described and were diffusely scattered throughout both organs. There was no sequestration but marbling could be made out. The histology was

typical of classical acute contagious pleuro-pneumonia. It is suggested that these lesions were due to minute fragmentation of the inoculum and consequent dissemination throughout the pulmonary capillaries, giving rise to multiple infarctions.

Remarks.

This test can be regarded as highly satisfactory since pronounced lesions were produced in nine out of the ten animals used. Taken in conjunction with the first experiment, it would appear as if the intravenous inoculation of agar emboli containing virulent culture or lymph is the method *par excellence* for producing the disease experimentally.

Before passing on to a general discussion, a short account will be given of an immunity test on vaccinated animals.

Immunity Test on Vaccinated Cattle.

The need of an efficacious method of testing immunity conferred by tail vaccination with attenuated culture has already been commented upon in this paper.

Now that the difficulty of reproducing the disease experimentally has been overcome, a ready method of testing post-vaccination immunity is available, and it was decided to carry out a preliminary test on a batch of vaccinated animals on the departmental Stock Farm.

Ten animals that had been tail vaccinated seven months previously with a 120th generation of pleuro-pneumonia culture were selected. All had been vaccinated at least twice, since prophylactic inoculation against this disease is carried out annually on the farm.

The agar emboli were prepared as described, shoulder lymph from a natural case of the disease being added to the agar.

No clinical or thermal reactions were reported, and it was decided to destroy all the animals in order to carry out a thorough examination of the thoracic viscera. In nine cases no lesions involving lungs or pleuræ were found. The exception was Cow No. 1,820, which was slaughtered 82 days after embolus inoculation. On opening the thorax a large area of adhesions, measuring 18 cm. by 6 cm. was observed covering the middle of the 6th to 9th ribs on the right side. Corresponding to this there was marked cicatrisation in the main lobe of the right lung. No sequestra were present.

On the left side there was a smaller area of adhesion attaching the central area of the left cardiac lobe to the middle third of the 6th and 7th ribs. The lung was free from lesions.

General Discussion and Conclusions.

It will be noted that in experiments involving the use of 20 cattle, 14 cases of contagious pleuro-pneumonia have been produced by the embolus method, while the other six resisted infection.

This is easily the largest successful transmission experiment on record and confirms the opinion of Daubney and others that there is now available a ready method of investigating the many obscure points concerning the pathology and transmission of this disease.

Further, it has been shown for the first time that post-vaccination immunity can be tested by this method. Although there were only ten animals in this experiment, results were highly encouraging, for only one showed lesions at *post-mortem*. This compares very favourably with the result of experiment No. 2 in which nine out of ten susceptible showed very marked lesions. It is reasonable to conclude that the vaccine issued by this laboratory for prophylactic inoculations in the field possess valuable immunising properties, though, statistically, it is desirable that many more vaccinated animals should be tested. This will actually be undertaken at the laboratory in the near future.

It should be noted that the vaccine used on the subjects of the test was a 120th generation subculture. From experience it has been found that it is dangerous to use any younger generation for field use in Nigeria, though in other parts of tropical Africa, *e.g.*, Gold Coast, reactions with a generation over the third or fourth " are exceptional " (Stewart).* In the Sudan, according to Bennett, the 7th generation may be used with safety.

It would thus seem as if the cattle of Nigeria are more susceptible to the disease than are the cattle of the Gold Coast or the Anglo-Egyptian Sudan though, in the opinion of some experienced workers on this disease, the possibility of plurality of strains cannot even now be altogether excluded.

Further experiments are being initiated to determine by the embolus method (*a*) at what period after vaccination immunity is established, (*b*) the duration of immunity conferred by ordinary tail vaccination as practised in Nigeria, and (*c*) at what generation the vaccine ceases to possess any immunising value. Information on this latter point is very important because it has been found from experience in Nigeria that vaccines younger than 120th generation may occasionally give a more severe local reaction than is desirable, and that somewhere in the neighbourhood of the 185th generation the vaccine is no longer efficacious.

How exactly the embolus method acts has not yet been worked out in detail, and the subject is worthy of further research since it may throw light on the pathogenesis of the disease under natural conditions.

It is generally assumed that in nature infection is aerogenous and that the virus reaches the lungs along the peribronchal connective tissues. Probably in the case of a successful transmission by an agar embolus the virus travels along the perivascular tissues.

Conditions for the proliferation of the virus in the lungs are ideal. The embolus, firmly lodged in a vessel of the pulmonary system, acts

* Private communication.

like a culture medium and produces enormous quantities of virus which passes into surrounding lung tissue.

The question as to whether mechanical injury to the lungs is a factor that has not received due consideration in previous attempts at transmission by ordinary means is suggested by the fact that simple intravenous inoculation of lymph or virus consistently fails to produce lung lesions, whereas if the virus is introduced into the blood stream in some formed substance, *e.g.*, agar it produces typical lesions of the disease. The average size of the emboli used in our experiments was 80 by 8 mm. and it is inconceivable that they did not cause some very material injury to the lung tissue as the result of their impaction in an artery of no mean size.

Turner, Campbell and Dick (1935) have reported that they can produce the disease by intravenous inoculation of ground up lung tissue without the addition of any agar. No information is given as to the fineness of the " ground up " tissue used.

Further experiments are being planned to determine whether injury to the lung tissue is a necessary factor for the development of the characteristic lesions of the disease.

Certain facts emerge from the experiments in this paper which may be briefly summarised as follows :—

1. The intrajugular inoculation of agar plugs containing lymph or culture is an effective and rapid method of producing, experimentally, lesions of contagious pleuro-pneumonia. In susceptible cattle there are often well-marked clinical symptoms following a period of incubation which averages one week. In more resistant animals symptoms are vague or absent even though there may be well-developed lesions in the thorax.

2. Sequestration and encapsulation may be complete in as short a period as 15 days after the appearance of clinical symptoms. On the other hand they were considerably delayed in one animal (Bull No. 1,800 in experiment No. 1), in which after 74 days there were no signs of the development of these lesions. Altogether, sequestration was found in seven out of twelve cases, but judging from puckering and depressed areas of cicatrisation seen in certain lungs there is the possibility that in a number of cases the sequestra had healed out.

3. In five animals, Nos. 1,800, 2,070, 2,073, 2,077 and 2,078, the lungs alone were the site of lesions, while in seven cases, Nos. 1,711, 1,803, 1,427, 1,799, 2,072, 2,071 and 2,075, both the lungs and pleuræ were involved. In many of the more chronic cases a fibrinous pleurisy was the only evidence of former infection.

4. No lung lesions were found in the two animals which received virus into the thoracic cavity though there was an extensive fibrinous pleurisy. This confirms earlier work in showing that the lungs escape infection when the pleural cavity is primarily affected. No satisfactory explanation of this phenomenon has yet been offered.

5. The nature of the lesions in all the cases in experiments Nos. 1 and 2, except possibly No. 2,078, which was seriously ill when

destroyed, suggests that recovery would have occurred if the animals had not been slaughtered for experimental observation. It appears to be a reasonable assumption that in this country a considerable percentage of animals survive infection and of these a large number becomes " lungers."

6. The vaccine test, although used on comparatively few animals, proved satisfactory and deserves a more extended trial. Out of the ten test animals only one showed evidence of having reacted to the inoculation of embolus containing virus. In two control tests, five out of ten respectively showed extensive lesions of contagious bovine pleuro-pneumonia.

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IMMUNISATION OF BIRDS AGAINST FOWL POX AND PIGEON POX RESPECTIVELY WITH VIRUSES PROPAGATED ON THE CHORIO-ALLANTOIC MEMBRANE OF THE DEVELOPING EGG.*

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As a result of investigations undertaken during the past fifteen years, the protection of poultry against infection with fowl pox virus has been placed on a fairly satisfactory basis. Nevertheless, the measures which are currently employed are not always entirely durable and efforts are still being made to devise more efficient methods of immunisation.

In this country Doyle (1930[a], 1930[b], 1934) has completed numerous laboratory and field experiments with a virus maintained in the pigeon and has clearly shown that fowls inoculated with unattenuated pigeon pox virus are solidly immunised against natural infection with fowl pox. His results have been partly confirmed by other workers (Zwick, Seifried and Schaaf, 1928; Johnson, 1931; Lerche, 1931; Stafseth, 1931; Glover, 1931; Kligler, Komarof and Fiat, 1933, etc.) but opinions differ regarding the exact duration and strength of the immunity. Thus Broerman and Edgington (1932), Michael (1932), Brunett (1933), Johnson (1934), Lubbehusen and Ehlers (1934), etc., assert that although pigeon virus does produce some degree of protection, it is only relatively efficient as an immunising agent.

Johnson (1934) in common with other American workers, has reported favourably on vaccination with desiccated fowl pox virus. The vaccine, prepared from comb lesions which have been dried and stored at a low temperature, is applied to a few feather follicles on the leg. It is claimed that this method confers a high degree of immunity. Doyle (1934) agrees that pigeon pox virus may fail to give complete protection against a severe artificial infection with fowl pox virus, but claims that very extensive field trials have shown that it confers a solid immunity against gross natural infection. He rightly draws attention to the undoubted danger of using unattenuated fowl pox virus, partly because immunisation by this method is achieved at the risk of perpetuating infection in the flock in which it is used, and partly because it is not always devoid of risk to the vaccinated birds. He considers that many of the poor results which have been obtained with pigeon pox virus can be attributed to the indifferent manner in which it has been prepared or applied.

In a previous series of experiments (Glover, 1931) it was noted that unmodified fowl pox virus applied to the feather follicles of the leg sometimes induced severe generalised lesions and was, therefore, unsuitable for protective inoculation on a large scale. On the other hand, pigeon pox virus applied under laboratory conditions protected fowls against a severe contact infection. Subsequent experiments indicated, however, that the duration of the immunity, both in regard to length and solidity, was subject to marked variations. In some

*These experiments were commenced at the Institute of Animal Pathology, Cambridge, and were completed at the National Institute for Medical Research, Farm Laboratories.

instances, birds vaccinated with pigeon pox and subsequently exposed to infection, showed moderate lesions of fowl pox within two months of receiving pigeon pox vaccine, while unfavourable weather conditions undoubtedly lowered the resistance of protected birds.

It is suggested, therefore, that while pigeon pox virus provides an immunising agent of considerable value in the field, there is still room for improvement. The present series of experiments was undertaken with the object of studying the possible value of fowl and pigeon strains cultivated on the developing egg as immunising agents.

The first reported success on the cultivation of fowl pox virus in tissue culture was that of Findlay (1928), who used the technique of Carrel and Rivers (1927) for vaccinia virus. Berkefeld filtrates mixed with embryo chick tissue were implanted in flasks with chicken plasma. The strain was carried through four generations. Later, Findlay's experiments were confirmed (Glover, 1929) with pigeon pox and a strain of this virus was carried through five generations. In order to secure active multiplication of the virus the brain or skin of embryo chicks were found to be more suitable than other tissues. In the meantime Woodruff and Goodpasture (1931) had developed a technique for the propagation of viruses on the chorio-allantoic membrane of the hen's egg and were successful in adapting fowl pox virus to this tissue. Burnet and Lush (1936), Burnet (1936) subsequently studied the reactions of fowl pox virus and Kikuth's canary virus which is closely related to fowl pox, on the egg membrane. No difficulty was experienced in adapting these strains and thus obtaining abundant amounts of bacteria-free, virus suspensions. Burnet (1936) suggests that virus grown on the egg might provide suitable material for vaccination against fowl pox. This is a possibility which has recently been explored by Brandley (1936). The method adopted was to triturate a fresh membrane suspension in 20 per cent. glycerol and apply a few drops to the feather follicles by the " stick " method. Immunity tests performed 25 to 65 days later on the opposite leg using a potent suspension of infective comb lesions showed that the protection was as solid as that induced by natural virus. Boley (1937) suggests drying the membranes *in vacuo*, suspending in glycerol and standardising on chicks by serial dilutions.

Cultivation of Fowl Pox Virus and Pigeon Pox Virus on the Chorio-allantoic Membrane.

One strain of pigeon pox and two strains of fowl pox virus have been successfully propagated on egg membranes. The pigeon virus has been described in a previous communication (Glover, 1929). It is sufficient to note that it was originally derived from the pigeon and was not a fowl strain which had been artificially adapted to this species. It has been passed through pigeons at regular intervals for ten years and shows no evidence of mutation in respect of the type of lesion which it produces in the pigeon and fowl respectively. The two fowl strains were of full virulence. One was isolated at Cambridge (I.A.P. 3) and the other was obtained from the Wellcome Physiological Research Laboratories (T.V. 6). As both gave identical

lesions on egg membranes, strain I.A.P. 3 was abandoned, the main investigation being confined to the second strain T.V. 6.

The method used by Burnet (1936) for the inoculation of the developing egg has been closely followed. As it has been fully described in his monograph on this subject, it is unnecessary to give details of the procedure.

Method of Initiating Cultures.—In each instance the initial inoculum was a suspension of an infected follicle collected from the under surface of the skin in the manner described in the previous article. It may not be out of place to mention that this method has been employed during the past eight years with uniform success and no trouble has been experienced with bacterial contaminants. It was with some surprise, therefore, that a statement was noted in a recent article by Baudette and Hudson (1938) to the effect that, while this method is useful for fowl pox it is unsuitable for pigeon pox, since unfiltered seed material was always heavily infected with various bacteria. A satisfactory explanation of their results is not obvious. It is possible that these authors, who were working with chickens, may have used pigeon virus initially contaminated with organisms which were transferred from passage to passage. Our own pigeon pox virus which is thoroughly dried *in vacuo* after each passage, has never given any difficulties in this direction.

Lesions produced on the Chorio-allantoic Membrane.

If a strong suspension of pigeon pox virus is applied to the membrane discrete lesions consisting of numerous, slightly raised, whitish ares are visible by the 24th hour. They rapidly increase in size and by the 48th hour have almost completely coalesced. With further incubation multiplication continues the membrane presenting a uniform, milky white appearance ; it also becomes much thickened and œdematous. At the third day it is often five to six times the normal in thickness and is almost completely opaque with a mottled, irregular surface. The embryo is not killed ; indeed, in a high percentage of cases, the chick will hatch if the egg is maintained in an incubator with a suitable degree of humidity. In view of the enormous thickening and distortion of the chorio-allantoic membrane, the resistance of chick embryos in infected eggs is most remarkable. With diluted suspensions of the virus the changes naturally develop more slowly and discrete lesions are seen up to the 4th day ; nevertheless, unless minimum infective doses have been injected the lesions eventually coalesce and the ultimate stage is one of uniform œdema and thickening of the membrane. The impression has been gained that pigeon pox represents the most proliferative type of virus for the egg membrane which has yet been studied.

With fowl pox virus, on the other hand, although some degree of proliferation is seen there is a greater tendency to necrosis with hæmorrhage. Membranes inoculated with a strong suspension of virus show a slight but uniform thickening at the third day and

are covered with a thin layer of necrotic material with small hæmorrhages. As the embryo usually dies between the third and fourth days, there is a cessation in the proliferative type of change and the marked increase in thickness which occurs with pigeon pox is entirely lacking. Burnet (1936) has noted that with two English fowl pox strains the lesions were discrete and the membranes œdematous and opaque. Histologically, there was no necrosis of the affected ectodermal cells and consequently little inflammatory reaction. In this respect they showed a marked difference as compared with membranes affected with vaccinia virus. An Australian virus, on the other hand, which at first behaved as the English strain, underwent mutation after serial passage and eventually produced lesions which much resembled the vaccinal reaction. The fowl strains which we have investigated behaved at first as the English strains examined by Burnet, but after twelve passages the lesions became more necrotic and less proliferative in nature, whilst by the twentieth passage there was an obvious decrease in the degree of œdema. Discrete lesions were obtained with dilutions up to 1 : 100,000 but with stronger suspensions, *e.g.*, 1 : 100 to 1 : 5,000, the whole of the surface was covered with a thin fibrino-necrotic layer and there were also distinct hæmorrhages. Both the pigeon and fowl strains were transmissible to duck eggs where they induced the formation of changes which closely resembled those occurring in hen eggs.

The infectivity of the egg passage strains was checked from time to time on appropriate test animals. A portion of a membrane infected with a 1 : 100 dilution of the virus and incubated for a period of three to five days was weighed and then rubbed up in sterile broth. Serial dilutions were prepared and applied to the plucked breasts of pigeons or young fowls. The results are shown in Tables I and II.

Behaviour of Viruses after Repeated Passage on Egg Membrane.

One of the objects of these experiments was to determine whether the propagation of these strains on the chorio-allantoic membrane would induce mutations in these viruses. It was conceivable that if such changes occurred they might be either advantageous or detrimental from the point of view of vaccination. For example, fowl pox virus as a result of repeated passage might become enhanced in virulence and therefore even less suitable for vaccination than the natural material obtained from comb or leg lesions. On the other hand, the effect of egg cultivation might operate in the reverse direction and produce a progressive decline in virulence such as has been reported with other diseases. Similar changes might also take place with pigeon pox virus after prolonged cultivation on the egg membrane. The pigeon strain with which we have been working has invariably produced extremely well marked local lesions in pigeons but has never generalised, while the response in the fowl has always been confined to a swelling of the inoculated follicles. It was expected

Table I.

Infectivity of Egg Propagated Pigeon Virus.

Generation	Species	1/100	1/1000	1/10,000	1/100,000	Intravenous.
1	P	++++	+++	+	—	0
5	P	++++	+++	++	+	0
10	P	++++	+++	+++	+	0
	F	+++	++	? +	—	0
16	P	+++	+++	++	?	0
20	P	+++	+++	++	+	—
	F	+++	+++	++	?	—
30	P	✱+++	✱++	✱+	?	0
38	P	✱++	✱++	✱+	?	0
	F	++++	+++	++	+	—
44	P	✱++	✱++	—	—	—
50	P	✱+	✱+	✱+	—	0
	F	+++	+++	++	?	

P = pigeon.
F = fowl.
✱ = marked decline in degree of follicular reaction. Scabs formed rapidly.

++++ = severe reaction of follicles.
+++ = fairly severe " " "
++ = moderate " " "
+ = slight " " "
— = no " " "
0 = not done.

Table II.

Infectivity of Egg Propagated Fowl Virus.

Generation	Species	1/100	1/1000	1/10,000	1/100,000	Intravenous.
1	F	+++	++	?	—	S.G.
5	F	++++	+++	++	+	S.G.
15	F	++++	+++	+++	+	S.G.
	P	—	—	0	0	0
23	F	✱++	✱++	✱+	—	M.G.
32	F	✱+	✱+	✱+	—	M.G.
	P	—	—	0	0	—

S.G. = severe lesions comb, tongue and palate.
M.G. = moderate " " " " "
✱ = decline in severity of reaction.

that in the case of the pigeon virus the most likely mutation would be a progressive decrease in virulence for the pigeon and an increase for poultry.

In order to detect such changes in the two viruses after passage on egg membranes both strains had been tested on fowls and pigeons at comparatively frequent intervals. The results are shown in Tables I and II.

With regard to the fowl strain, the impression has been gained that there has been a slight but nevertheless appreciable decline in virulence. The methods adopted for testing the infectivity have been two in number, *viz.* : (1) the application of serial dilutions to the comb or to the feather follicles of the leg, and (2) the intravenous inoculation of a 1 : 100 dilution. No evidence of mutation was obtained until the 15th transference, but between the 15th and 30th passages the lesions produced on the skin and the comb were less severe and showed a tendency to rapid scab formation and resolution. Similarly, the lesions induced on the comb, tongue and palate following the intravenous inoculation of the virus, were not so severe or prolonged as those induced by natural comb material. The strain is being continued on egg membrane in the hope that with continued passage still further attenuation of the virus will be secured.

The pigeon strain which is now in its 50th passage shows recognisable changes in its character. When inoculated into pigeons the lesions induced on the de-plumed leg are not so marked ; moreover, there is less swelling of the follicles and the lesions tend to heal more rapidly. The reaction resembles to a marked degree the response obtained in the pigeon with a recently passaged fowl virus which has not been fully adapted to the pigeon. In the fowl, on the other hand, the strain shows no tendency towards a mutation and certainly has not yet acquired any of the characteristics of true fowl pox. After the 20th passage the degree of swelling of the feather follicles was slightly more apparent, but the increase in reaction was not great. At no stage have lesions been detected in the fowl other than at the point of inoculation. This is a point of particular importance and has been watched with especial care. From the 20th passage fowls were inoculated at regular intervals by the intravenous route, a mode of infection which in our experience has been extremely valuable in separating strains of average virulence from those which are attenuated. In the former instance diphtheritic lesions are invariably formed on the soft palate, tongue and comb, especially in young birds, and death frequently occurs about the 14th day. Attenuated strains fail to produce generalised lesions. So far the pigeon strain passed through the hen egg has proved incapable of producing generalised lesions.

The Resistance of Virus.—Both fowl pox and pigeon pox viruses under suitable conditions are relatively resistant and can be kept for a considerable length of time. It has already been noted (Glover, 1931) that the scabs from pigeon pox lesions dried in the whole state

retained their virulence in the refrigerator for many months, although loss in infectivity was reported when they were ground to a fine powder. A recent examination of stored batches of pigeon virus has shown that potency is not entirely lost after two and a half years. On the other hand, membranes from eggs infected with both fowl and pigeon strains, when dried and stored under similar conditions, showed a fairly substantial loss in potency after a few weeks. Several experiments were undertaken with membranes stored in buffered glycerol solution, broth, etc. In each case, approximate estimates of the end points in infectivity were made. It is not proposed to furnish details of all the results obtained, but it may be mentioned that, to our surprise, the membranes appeared to conserve their potency much more readily in broth than in any other substrate which was tried. This is shown in Table VIII which sets out the results of titrating the seventh egg passage of pigeon pox virus at intervals. Portions of infected membrane measuring about 0·5 cm. were stored in various fluids which were kept in the refrigerator. Weighed amounts were then titrated on pigeons. It will be noted that virus kept in broth had not entirely lost its potency after 380 days, whereas virus in other fluids became inert between the 93rd and 297th days.

Experiments on Immunity.

The experiments on immunity were undertaken with the object of determining firstly, whether egg propagated virus was as efficient an immunising agent as natural tissue, and secondly, whether it would be possible to use such material for improving the durability of the immunity induced by current methods, *i.e.*, the single inoculation of a pigeon or fowl strain.

It has already been mentioned that pigeon pox virus does not give as solid or lasting an immunity in poultry as fowl virus. It may be stated at once that in so far as its immunising efficiency is concerned, egg propagated pigeon virus in a single dose has shown no clear-cut improvement over natural virus. The possession of artificially grown strains, however, has opened up methods of protection which have not yet been assayed, *viz.*, increasing the degree of immunity by re-inoculation. It is obvious that the intradermal route is not suitable for reinforcing the immunity since the protection induced by a primary stimulus is sufficient to prevent " takes " when a virulent suspension is again introduced into the feather follicles. This objection does not arise, however, when virus is injected subcutaneously, intraperitoneally or intravenously, but hitherto it has been difficult to apply these methods since suspensions of natural lesions cannot be used with safety on account of the complications arising from secondary organisms. Virus suspensions from egg propagated material are free from this drawback and they can be injected without danger by the different parenteral routes.

The experiments will be described under the following headings :

(*a*) The immunisation of pigeons with single or multiple doses of

pigeon virus ; (*b*) the immunisation of fowls with single or multiple doses of pigeon virus ; (*c*) the immunisation of fowls with pigeon virus followed by fowl virus.

Conduct of Experiments.—The vaccines, unless otherwise stated, were prepared from the supernatant fluids from 1 per cent. suspensions of three to five days egg membranes virus in saline broth lightly

Table III.

Immunising Effect of Single and Multiple Doses of Pigeon Virus in Pigeons.

Route.	Immunity Test, Interval in days.	Test dose, natural pigeon virus.
I.D.	21	1/50 —
S.C.	"	1/50 —
I.V.	"	1/50 —
I.P.	"	1/50 —
I.P.	"	1/50 —
—	Control.	1/10,000 +++ 1/50,000 +
S.C.	86	1/50 ++ 1/100 ++ 1/500 —
S.C.	"	1/50 +++ 1/100 ++ 1/500 —
I.V.	"	1/50 —
I.V.	"	1/50 —
I.P.	"	1/50 —
—	Control.	1/10,000 ++ 1/50,000 ?+
I.D.	174	1/50 ++ 1/100 —
S.C.	"	1/5[illegible],000 ++ 1/10,000 ++ 1/50,000 —
I.V.	"	1/50 ?+ 1/100 —
I.P.	"	1/50 + 1/100 + 1/500 —
—	Control.	1/10,000 ++++ 1/50,000 ++

I.D. = intradermal.
S.C. = subcutaneous.
I.V. = intravenous.
I.P. = intraperitoneal.

centrifuged for five minutes. The doses used were 1 c.c. subcutaneously, 1 c.c. intraperitoneally or 0·25 c.c. intravenously. The birds were tested for immunity after varying intervals, either by the application of serial dilutions of natural virus to the plucked breast (1:50 to 1:50,000) or by the intravenous injection of 0·25 c.c. of a 1:100 dilution.

(*a*) *Immunisation of Pigeons with Single and Multiple Doses of Pigeon Virus.*—A group of 14 pigeons was inoculated with a 1:100 saline broth suspension of the 13th egg passage of the pigeon strain.

Three groups of four each were injected by the subcutaneous, intraperitoneal and intravenous routes respectively, while the two which remained were infected by applying the virus to about a dozen follicles on the breast. In the latter a well marked eruption was produced in the follicles lasting for a period of about ten days. The introduction of virus by the intraperitoneal and intravenous routes was without visible effect, but a proportion of the birds injected subcutaneously presented slight swellings near the points of inoculation. The degree of protection was determined by the application of serial dilutions of natural pigeon virus as shown in Table III.

TABLE IIIa.

1st dose.	Interval (days)	2nd dose.	Immunity test Interval.	Test dose, pigeon virus.
I.D.	28	—	184	1/50 + 1/100 —
S.C.	"	S.C.	"	1/50 + 1/100 + 1/500 —
S.C.	"	S.C.	"	1/50 + 1/100 —
I.V.	"	I.V.	"	1/50 —
I.V.	"	I.V.	"	1/50 —
I.P.	"	I.P.	"	1/50 —
I.P.	"	I.P.	"	1/50 —
—	—	—	"	1/10,000 +++ 1/50,000 +
I.D.	28	—	276	1/50 —
S.C.	"	S.C.	"	1/500 ++ 1/1000 —
S.C.	"	S.C.	"	1/500 ++ 1/1000 ++ 1/5000 —
I.V.	"	I.V.	"	1/50 + 1/100 —
I.V.	"	I.V.	"	1/50 ?+ 1/100 —
I.P.	"	I.P.	"	1/50 ++ 1/100 ?+
I.P.	"	I.P.	"	died intercurrent infection.
—	—	—	"	1/10,000 +++ 1/50,000 ++

The most striking results were obtained by intravenous and intraperitoneal vaccination. After three months pigeons protected by these routes were completely immune, while at the sixth month the resistance was only just commencing to wane. The subcutaneous route, on the other hand, was much less satisfactory and by the sixth month the immunised pigeons were nearly as susceptible as the controls.

In the second experiment a group of 14 pigeons, which received an initial dose of egg membrane virus by intravenous, intraperitoneal, subcutaneous and intradermal routes respectively, was re-inoculated one month later with similar material (Table IIIa). The birds were tested for immunity after six and nine months respectively.

When compared with the birds which received a single dose, it is clear that a second injection of virus, one month after the first, had appreciably increased the resistance. As in the previous series, however, the subcutaneous route was less efficient than the intravenous or intraperitoneal routes.

It seemed to be of some importance to ascertain whether the virus was recoverable from different organs after intravenous injection; accordingly four pigeons were injected by this route and were killed after 10, 21, 50 and 75 days respectively. Suspensions were prepared from the livers, spleens and lungs and were tested on the breasts of normal pigeons. After 10 days virus was recovered from all three sites and was present in amounts which were just detectable in the spleen and liver after 21 days, but no reactions were obtained with any suspension after the 50th and 75th days. It would appear, therefore, that the virus disappears fairly rapidly from the tissues after intravenous injection.

In view of the interesting results obtained with certain viruses adsorbed on kaolin, alumina, etc., *e.g.*, foot-and-mouth disease, poliomyelitis, vaccinia, sheep pox, etc., it was decided to test the immunising effect of pigeon pox virus plus alumina. The method suggested by Tang (1932) for the preparation of the gel was followed. A series of preliminary experiments showed that the virus was readily removed from a suspension of infected egg membrane in distilled water by the alumina gel when the reaction of the mixture was acid. In one experiment 500 mg. of membrane (14th passage on egg) was ground up in 10 c.c. of sterile distilled water and lightly centrifuged. The supernatant fluid was added to 10 c.c. of standard alumina gel and sufficient saturated KH_2PO_4 solution was run in to bring the reaction to *p*H 4·2.* Six birds were then inoculated subcutaneously each with 1 c.c. of the suspension. The birds showed slight swellings at the point of inoculation which persisted for six weeks, but no skin reaction was observed. They were tested after two and four months respectively with the results shown in Table IV.

The results were most disappointing since the protection afforded by this method was not nearly so satisfactory as that resulting from a saline suspension.

(*b*) *Immunisation of Fowls with Single and Multiple Doses of Pigeon Virus.*—In view of the results obtained with the pigeon, it was decided that the experiments in the fowl should be confined to the intradermal and intravenous routes. It has already been mentioned that the egg membrane pigeon virus can be inoculated intravenously into the fowl without any apparent effect.

The first experiments were designed to compare the immunising effects of natural pigeon pox virus intradermally and egg membrane

*Experiments, which will be detailed in another paper, have shown that the virus is fixed to alumina at this *p*H but is not inactivated and can be eluted by changing the reaction in an alkaline direction.

pigeon virus intradermally and intravenously. A group of three month old brown leghorn chickens was selected and divided into four groups. The first was immunised by the application of a 1:100

TABLE IV.

PIGEON VIRUS ABSORBED TO ALUMINIUM HYDROXIDE.

Vaccine. (Subcutaneously).	Immunity test. Interval in days.	Test dose. Natural pigeon virus.
Virus + alumina.	45	(1/1000 ++ 1/50,000 + 1/10,000 —
	"	(1/1000 ++ 1/5000 ++ 1/10,000 —
	"	(1/500 +++ 1/1000 + 1/5000 —
	76	(1/1000 +++ 1/5000 ++ 1/10,000 —
	"	(1/5000 +++ 1/10,000 ++ 1/50,000 +
	"	(1/5000 +++ 1/10,000 ++ 1/50,000 ?+
Virus in saline.	45	1/50 ++ 1/100 ++ 1/500 —
	76	1/50 +++ 1/100 +++ 1/500 —
Control.	—	1/10,000 +++ 1/50,000 ++

dilution of natural pigeon pox virus to about one dozen follicles on the leg, while the second group was treated with a similar dilution of an egg membrane virus in its 34th generation. In each instance, there were well marked skin reactions of the usual type. The third group received 0·25 c.c. intravenously, while the fourth was reserved as controls. Equal numbers from each group were withdrawn at intervals and tested for immunity with a natural fowl pox virus either intradermally or intravenously. The results are shown in Table V.

It will be noted that after three weeks, the immunity in all groups was complete, while after three months, protection was satisfactory in both groups of intradermally vaccinated birds but was just commencing to break down in the intravenous group. At six months the immunity was definitely on the wane in each group particularly in the intravenously vaccinated birds. No difference was detectable as between natural and artificial virus applied intradermally. There was no question, therefore, that in so far as a single application was concerned, the intradermal route gave the most satisfactory results.

The next step was to ascertain the effect of giving an intravenous injection of egg membrane pigeon virus to fowls which had already been immunised by the intradermal or intravenous routes. As the subcutaneous method had given indifferent results in the pigeon, it was omitted in this experiment, but a few birds were included which had been immunised by the intramuscular route. The first inoculum was a 1:100 dilution of 32nd egg passage virus, and the doses were 0·25 c.c. by the intravenous route and 0·5 c.c. intramuscularly, while the reinforcing dose was a suspension of the 35th passage.

TABLE V.

FOWLS IMMUNISED SINGLE DOSE PIGEON VIRUS.

Vaccine.	Route.	Immunity test interval in days.	Route.	Test dose. Natural fowl pox virus.
*N.P.V.	I.D.	21	I.D.	1/50 —
"	I.D.	"	I.V.	—
*E.M.V.	I.D.	"	I.D.	1/50 —
"	I.D.	"	I.V.	—
"	I.V.	"	I.D.	1/50 - 1/100 —
"	I.V.	"	I.V.	—
—	—	"	I.D.	1/10,000 ++ 1/50,000 +
—	—	"	I.V.	S.G. Comb, tongue & palate.
N.P.V.	I.D.	95	I.D.	1/50 —
"	I.D.	"	I.V.	—
E.M.V.	I.D.	"	I.D.	1/50 —
"	I.D.	"	I.V.	—
"	I.V.	"	I.D.	1/50 ++ 1/100 ++ 1/500 —
"	I.V.	"	I.V.	sl. lesions, tip of tongue only.
—	—	"	I.D.	1/10,000 +++ 1/50,000 +
—	—	"	I.V.	S.G. comb, tongue and palate. died 17 days.
N.P.V.	I.D.	177	I.D.	1/100 ++++ 1/500 ++ 1/1000 ?+
"	I.D.	"	I.V.	M.G. comb and tongue.
E.M.V.	I.D.	"	I.D.	1/100 +++ 1/500 ++ 1/1000 —
"	I.D.	"	I.V.	M.G. comb and tongue.
"	I.D.	"	I.V.	M.G. sl. tongue, mod. comb.
"	I.V.	"	I.D.	1/5000 +++ 1/10,000 +
"	I.V.	"	I.V.	M.G. comb, tongue & palate.
"	I.V.	"	I.V.	M.G. " " "
—	—	"	I.D.	1/10,000 +++ 1/50,000 +
—	—	"	I.V.	M.G. comb, tongue & palate.

*N.P.V. = natural pigeon virus.
*E.M.V. = egg membrane virus.

The results are set out in Table VI. They show quite plainly that an increased immunity followed the double inoculation. An intradermal, reinforced by an intravenous injection, or two successive intravenous injections, gave almost equally good results. After five months, the birds were adequately protected, whilst the immunity

TABLE VI.

FOWLS IMMUNISED TWO DOSES PIGEON VIRUS.

1st dose Vaccine.	Interval (days)	2nd dose Vaccine.	Immunity test, Interval (days).	Route.	Test dose natural fowl pox virus.
I.D.	21	I.V.	28	I.D.	1/50 —
I.D.	"	I.V.	"	I.V.	—
I.V.	"	I.V.	"	I.D.	1/50 —
I.V.	"	I.V.	"	I.V.	—
I.V.	"	I.M.	"	I.D.	1/50 —
I.M.	"	I.M.	"	I.V.	—
—	—	—	"	I.D.	1/10,000 +++ 1/50,000 +
—	—	—	"	I.V.	
I.D.	21	I.V.	151	I.D.	1/50 —
I.D.	"	I.V.	"	I.V.	—
I.D.	"	I.V.	"	I.V.	—
I.V.	"	I.V.	"	I.D.	1/50 —
I.V.	"	I.V.	"	I.D.	1/50 ++ 1/100 + 1/500 —
I.V.	"	I.V.	"	I.V.	—
I.V.	"	I.V.	"	I.V.	—
I.M.	"	I.M.	"	I.D.	1/500 +++ 1/1000 ++ 1/5000 +
I.M.	"	I.M.	"	I.V.	Sl.lesions tongue & comb.
—	—	—	"	I.D.	1/10,000 +++ 1/50,000 ++
—	—	—	"	I.V.	M.G. comb & tongue. 20 days.
I.D.	21	I.V.	255	I.D.	1/50 + 1/100 ?+
I.D.	"	I.V.	"	I.V.	? sl. lesions comb.
I.V.	"	I.V.	"	I.D.	1/50 —
I.V.	"	I.V.	"	I.V.	—
I.V.	"	I.V.	"	I.V.	—
I.M.	"	I.M.	"	I.V.	M.G. mod. tongue, sl. comb.
—	—	—	"	I.D.	1/10,000 +++ 1/50,000 +
—	—	—	"	I.V.	M.G. comb and tongue.

after nine months was almost complete. As was the case with the subcutaneous administration of virus in pigeons, the intramuscular route was not satisfactory. This lack of protection was rather surprising and no adequate explanation of the poor response can be offered at the present moment.

TABLE VII.

FOWLS IMMUNISED PIGEON VIRUS FOLLOWED BY FOWL VIRUS.

1st dose	Interval (days)	2nd dose	Immunity test,* Interval (days)	Test dose natural fowl pox.	
				I.D.	I.V.
I.D.	21	I.V.	94	1/50 —	—
I.D.	"	"	"	1/50 —	—
I.D.	"	"	287	1/50[e]+ 1/100[e]+	v. sl. abortive lesion tongue.
I.D.	"	"	"	1/50[e]+ 1/100 —	—
I.V.	"	"	—	Died after test	dose
I.V.	"	"	—	" " "	"
I.V.	"	"	—	" " "	"
I.V.	"	"	—	" " "	"
I.V.	"	"	287	1/50 —	—
I.V.	"	"	"	1/50 —	—
I.D.	62	I.V.	103	1/50 —	—
I.D.	"	"	"	1/50 —	—
I.V.	"	"	"	1/50 —	—
I.V.	"	"	"	1/50 —	—
I.V.	"	"	"	1/50 —	—
—	—	—	"	(1/1000 +++	M.C. tongue & comb.
—	—	—	"	(1/5000 +	
I.D.	62	I.V.	181	1/50 —	—
I.D.	"	"	"	1/50 —	—
I.V.	"	"	"	1/50[e]+ 1/100[e]+	abortive lesion comb & tongue.
I.V.	"	"	"	1/50 —	—
I.V.	"	"	"	1/50 —	—
—	—	—	"	(1/5000 +++	M.C. tongue & comb.
—	—	—	"	(1/10,000 +	

*Combined intradermal and intravenous.
[e]Abortive lesions.
Controls Tables V & VI.

(*c*) *Pigeon Virus followed by Fowl Virus.*—The final experiment consisted in examining the effect of a reinforcing dose of fowl virus given to birds which had already received pigeon virus. A group of 24 three-month-old brown leghorns was used. The initial inoculation was a 1:100 suspension of the 34th pigeon pox generation given intradermally (twelve birds) or intravenously (twelve birds). Three weeks later one-half of each group received a second injection

of the 17th egg passage of fowl pox membrane by the intravenous route. An interesting phenomenon was encountered in this series. The birds, which had received the initial dose of pigeon virus by the intradermal route showed no apparent reaction to the intravenous injection of the fowl virus. Two were killed at the 5th and 10th days respectively and a suspension of mixed liver, spleen and lung applied to the breast of a normal bird. No reaction was observed. The birds prepared by the intravenous route, however, reacted in an entirely different manner. At the 48th hour they were dejected, refused to feed and seemed to have some difficulty in keeping on their feet. All except two died within ten days. No lesions of fowl pox were noted, but in each instance the liver was enlarged and pale. Sections showed a slight lymphocytic infiltration and some evidence of vacuolation

TABLE VIII.
CONSERVATION OF EGG MEMBRANE PIGEON VIRUS (7th PASSAGE).

7th Passage, 3-7-37.	36 D.	72 D	93 D	187 D	297 D	380 D.
Dried.	1/1000	1/10	—	0	0	0
Broth pH 7.4, unsealed.	1/10,000	1/10,000	1/1000	1/100	1/100	Undil.
Broth pH 7.4, sealed.	1/1000	1/100	undil.	—	0	0
Glycerol Broth 7.4	1/10,000	1/100	1/10	undil.?	—	0
Glycerol phosphate buffer, 7.4	1/5000	1/1000	1/10	—	—	—
Glycerol borate buffer, 7.4	1/10,000	1/5000	1/500	1/10	—	—

Titre before storage 1/50,000 +

of the liver cells, with a little loss in staining affinity of the nuclei. A normal bird which received an emulsion of mixed organs from one of these fowls which died, remained healthy and showed no skin eruption.

The birds which remained were inoculated intravenously with fowl virus after an interval of two months without untoward effect. The immunity tests were applied to small groups by the simultaneous application of falling dilutions of natural virus and the intravenous injection of 0·25 c.c. (Table VII).

The results were quite satisfactory. Birds receiving an intradermal followed by an intravenous dose of vaccine at an interval of 21 days were completely immune at 94 days. After 278 days, very slight abortive lesions were noted on the breast while one bird showed a transient lesion on the tongue which quickly healed. Only two birds remained in the group inoculated with two intravenous doses at

21 days interval; both were completely resistant at the 287th day. In the batch which received the reinforcing dose at the 62nd day, there was absolute protection to a test dose administered after 103 days, while after 181 days, with the exception of very slight abortive lesions in one bird, there was no evidence of any detectable decline in the immunity.

Discussion.

The method of immunising poultry against fowl pox by applying a pigeon pox virus to the feather follicles of young chicks, which is being applied on a large scale in this country, is simple, safe and, on the whole, reasonably efficient. Nevertheless, the degree of immunity which is obtained does not always afford a protection of adequate duration, and fairly severe natural outbreaks of the disease sometimes arise in flocks which have been vaccinated by this method. Attempts have been made to overcome this difficulty by using strains of fowl pox instead of pigeon pox virus, but available evidence suggests that such material is always a potential source of danger and must inevitably give rise to anxiety lest the vaccine itself should set up severe and uncontrollable lesions.

The successful propagation of fowl and pigeon pox viruses in the developing egg has opened up new possibilities and enabled the problem to be approached from more than one angle. The present studies, which amply confirm the results of other workers, indicate quite clearly that both these viruses can be propagated serially through the egg. From the quantitative point of view, this method of maintaining the virus does not appear to lead to any appreciable loss in potency; but qualitatively it would seem that the viruses may be undergoing modifications of some importance. Thus, in the case of pigeon pox, changes in the characters of the skin lesions induced in the pigeon were noted after the 16th passage, and there was an obvious difference, in the form of a decline in the degree of reaction between the follicular lesions produced by natural virus and those resulting from the later egg passage strain. In the fowl, on the other hand, the dermal lesions induced by the pigeon virus have not shown any degree of attenuation and are, in fact, slightly more pronounced. It is important to note, however, that no tendency towards the production of generalised lesions has yet been detected. It seems legitimate to infer, therefore, that repeated passage through the egg has made an impression on the pigeon strain, but there is no indication that it will produce other than strictly localised lesions in the fowl.

Fowl pox virus, on the other hand, seems to become less infective for poultry when transmitted through the egg. The particular strain with which we have worked has not yet reached the stage where it fails to produce lesions of generalisation, but at the 35th passage it did not induce such severe lesions as the parent material,

The actual experiments on immunity require little comment. The number of birds which has been used is relatively small, but this has been deliberate. It might have been possible to vaccinate comparatively large numbers of poultry and subsequently to expose them to natural infection, but previous experience has shown that, according to the circumstances, the proportion of unprotected birds which contracts the disease is most irregular. It was considered, therefore, that more satisfactory information would be obtained by restricting the numbers and by determining the exact degree of resistance by the inoculation of graded doses of virus.

The impression has been gained that egg propagated pigeon virus is quite suitable for the immunisation of both the pigeon and the fowl, and when applied by the dermal route seems to give good results, which are no less satisfactory than those induced by natural virus. This type of material possesses the additional advantage that the basal immunity induced by a primary injection can be reinforced by a parenteral inoculation of this bacteria-free suspension. The field experiments already undertaken in America by Brandley, Boley and others with egg propagated virus, are encouraging and it is hoped that crucial tests may be applied in this country to determine whether this artificial virus can be used to protect poultry which may be exposed to infection.

Conclusions.

(1) A strain of pigeon pox virus has been maintained on the chorio-allantoic membrane of the hen's egg for 50 generations. The virus has become slightly attenuated for the pigeon, but induces good dermal lesions in the fowl. It shows no signs of enhanced virulence for poultry. Similarly, a fully virulent fowl pox strain has been carried through 38 generations. It still fails to induce lesions in the pigeon and shows some evidence of attenuation for the fowl.

(2) Membranes infected with pigeon virus can be stored in broth at a temperature of 0 to 4° C. There is a gradual loss in infectivity, but lesions are still obtainable in the egg, and in the pigeon after 380 days. Fowl virus is also relatively stable under similar conditions.

(3) Pigeons which have received a single dose of egg membrane virus by the intradermal, intraperitoneal or intravenous routes show a fairly durable immunity which can be reinforced by a second dose of virus. The subcutaneous route is less effective. In fowls the intradermal application of the egg virus appears to be as satisfactory as natural virus for the immunisation of the fowl, but the immunity shows signs of waning after about six months. The immunity can be reinforced by a second injection of pigeon or fowl virus given intravenously or intraperitoneally. There is a slight indication that fowl virus is the more effective.

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EXPERIMENTS IN CALVES ON IMMUNITY CONFERRED BY SINGLE AND DOUBLE INJECTIONS OF BCG IN AN OILY EXCIPIENT*

By J. B. Buxton and R. E. Glover.

Introduction.

In an earlier report (Buxton and Glover, 1934) we published the result of an experiment in calves performed with the object of determining whether it was possible to induce an immunity against tuberculosis by the subcutaneous inoculation of (*a*) BCG vaccine, and (*b*) dead tubercle bacilli, each suspended in olive oil or liquid paraffin after coating with gum acacia solution. In so far as killed tubercle bacilli were concerned, the results were not particularly encouraging since the degree of immunity which was obtained was of a low order; moreover, the reactions to the dead vaccines were too severe to warrant their employment under practical conditions. It should be pointed out, however, that comparatively large amounts of killed organisms were used (0·5 gramme to 1·0 gramme) and that different results might have been obtained with smaller quantities. In the case of BCG a satisfactory degree of immunity was induced. The protected calves, four in number, were tested by the oral administration of virulent tubercle bacilli six months after vaccination. At autopsy, three months later, three out of the four were completely immune, while in the fourth the lesions were quite trivial.

As a result of this preliminary experiment, the opinion was formed that of the two excipients, olive oil gave better results than liquid paraffin. It was decided, therefore, that a larger series of calves should be tested with BCG in olive oil, further experiments on dead vaccines being deferred for the time being.

Animals Used in the Experiment.

A group of 27 three to four-months-old calves was obtained from tubercle-free herds in Ayrshire. On arrival at the Institute, they were immediately isolated and were then tuberculin-tested with synthetic medium tuberculin by the double intradermal method. The initial test failed to reveal any reactors, while two subsequent tests which were applied at approximately two monthly intervals were also completely negative. During this period, three of the animals died from intercurrent diseases, leaving 24 available for the main experiment. It was originally intended to sub-divide these into three groups of eight each (six vaccinated and two controls) which were to be tested

*The experiments recorded in this paper were carried out at the Institute of Animal Pathology, Cambridge.

with virulent tubercle bacilli at six, nine and twelve months respectively after vaccination. During the period intervening between the vaccination and the testing of these animals, four succumbed from advanced parasitic gastro-enteritis. It was decided, therefore, to modify the experiment and to allocate the animals to two groups, *viz.* (*a*) Seven vaccinated and two controls to be exposed to infection six months after vaccination, and (*b*) eight vaccinated and three controls to be infected when the results of the first experiment became available.

A.—Group I.

(*Seven Vaccinated and Two Controls.*)

Preparation of the Vaccine.—The method of preparing the olive oil vaccine was fully described in the previous article (Buxton and Glover, 1934). Briefly, the BCG strain was seeded on to Herrold's egg yolk agar containing 2 per cent. of glycerol. The growth from eight to ten day-old cultures was then washed off in a small quantity of 10 per cent. gum acacia solution and was shaken for two hours with beads. The quantity of organisms per c.c. was determined by centrifuging a measured amount and weighing the sediment. Two suspensions were prepared containing 5 mg. and 50 mg. per c.c. respectively, each of which was completed by the addition of 4 parts of sterile olive oil to 1 part of gum acacia. On May 1st, 1936, three calves (406, 414, 427) were inoculated in the subcutaneous tissues of the brisket each with 5·0 c.c. of the 5 mg. suspension, and four (385, 402, 420, 428) in a similar manner with 50 mg. Six guinea-pigs were also inoculated subcutaneously each with 0·5 c.c. of the 50 mg. suspension and were killed four months later. There were fibrous nodules containing caseo-pus at the site of the injection, but there were no lesions elsewhere.

Local Reactions.—The local lesions in the calves receiving the 5 mg. doses followed approximately the same course. Twenty-four hours after injection there was a severe reaction, the subcutaneous tissues at the point of inoculation being tense and hot. During the succeeding seven days, the swelling which at first was ill-defined and diffuse, tended to resolve itself into a circumscribed nodule about 5 cm. by 8 cm. in size. This stage was followed by a steady retrogression of the lesion ; by the third month, the swelling had shrunk to the size of a small bean and by the fifth month was no longer palpable. At *post-mortem* examination, no lesions were discoverable in any of the animals.

The reactions of the 50 mg. doses were rather more severe. In two animals, a dense swelling measuring 6 mm. to 8 mm. was readily palpable up to the fifth month ; thenceforward, it slowly retrogressed in one calf, but persisted in the other. In the remaining two, the lesions became steadily smaller in size and by the fifth month were just discernible as bean-like, firm swellings. In three out of four no

lesions could be found at autopsy, while in the fourth there was a dense fibrous swelling 4 cm. by 6 cm., in the region of the brisket.

Tuberculin Tests.—Following the injection of the vaccine, the calves were subjected to periodic tuberculin tests. In each instance they became strongly allergic shortly after the inoculation, but by the third month the sensitivity towards tuberculin commenced to decline. Nevertheless, the animals continued to give positive reactions until the termination of the experiment. There was no increase in responsiveness as a result of the administration of the test dose of virulent organisms.

Immunity Test.—On October 14th, 1936, *i.e.*, approximately six months after the calves had been vaccinated, the seven protected animals together with the two controls (Nos. 390 and 407) were tested for immunity by the oral administration of 5 mg. of virulent tubercle bacilli in 2 c.c. of saline. The test dose comprised six bovine strains which were grown on egg for ten days and were then washed off in normal saline solution. As in previous experiments, the suspension was pipetted into the mouth. Before receiving the test dose the two controls had been tuberculin tested on three occasions with negative results ; after receiving the test dose each one gave a reaction on the 27th day.

Control Animals.

Calf No. 407, was killed on April 5th, 1937, *i.e.*, 173 days after the test dose.

Post-mortem findings.— The right pharyngeal gland was enlarged by one-half the normal size and was beset with caseous tubercles too numerous to count and varying from a pin's head up to aggregates from 3 to 5 millimetres in size. The left gland contained an aggregate of four millet seed sized tubercles and a single lesion the size of a pin's head. The mesenteric glands were moderately severely affected, the lesions being fairly evenly distributed. In all, 32 foci were counted, and they varied from small caseous lesions the size of a millet seed up to caseo-calcareous nodules measuring 2 cm. One gland in the ileo-cæco-colic group contained two aggregates about 1·5 cm. in size. The bronchial and smaller mediastinal glands were free from visible lesions, but the large mediastinal gland contained three caseo-calcareous aggregates up to 1·5 cm. in diameter and five discrete tubercles. There were no lesions in the lungs or in any of the other organs or glands.

Two guinea-pigs inoculated with a suspension from the affected mediastinal glands died of generalised tuberculosis in 63 and 86 days respectively.

Calf No. 390 was killed on April 16th, 1937, *i.e.*, 184 days after the test dose.

Post-mortem findings.—There were no visible lesions in the glands of the head. Extensive caseo-calcareous lesions were regularly distributed throughout the chain of mesenteric glands. In all,

about 50 foci were counted, the majority of which consisted of aggregates of tubercles up to the size of a french bean. Five glands in the ileo-cæco-colic group were also affected. There were no lesions elsewhere.

Two guinea-pigs were inoculated with a suspension from an affected mesenteric gland. One died of generalised tuberculosis at the 70th day, and one was killed *in extremis* at the 83rd day. Both showed generalised tuberculosis.

Vaccinated Animals.

The three calves which received the 5 mg. dose of vaccine were killed at the 175th, 177th and 183rd day respectively after the immunity test. Nos. 414 and 427 showed fairly severe lesions in the mesenteric glands similar in extent to those seen in control 407. In 406, both pharyngeal glands were affected while foci varying from a pin's head up to aggregates of 0·5 cm. in size were found in the mesenteric glands.

The four calves injected with the larger dose of vaccine were killed at the 174th, 180th, 182nd and 188th day respectively. They were much less severely affected than the controls. In three animals (385, 402, 428) the lesions were confined in each case to a single gland in the alimentary tract. The foci were definitely enclosed in a thick fibrous tissue capsule suggesting that they were undergoing resolution. In the fourth calf (420) there was a single caseous lesion about 0·5 cm. in size in the right pharyngeal gland and caseo-calcareous lesions, varying in size from a millet seed to aggregates of about 1 cm. in diameter, in five mesenteric glands. (Table I.)

Guinea-pigs which were inoculated with suspensions from affected glands from each of these animals died of generalised tuberculosis within two or three months.

Summary.—The experiment which has just been described was most disappointing since it failed to confirm the results which had been obtained in the previous series where three out of four calves were completely immune after receiving BCG in oil. It is true that in the present experiment the lesions in the calves injected with 50 mg. doses were less extensive than in the two controls, but those animals which received 5 mg. were almost as severely affected as the latter. In any case, the results were unfavourable when compared with a similar series of calves which were protected with BCG by the intravenous route and were also tested by the oral administration of virulent cultures six months later (Buxton, Glover and Griffith, 1934).

B.—Group II.

(*Eight Vaccinated and Three Controls.*)

In view of the indifferent results obtained in the preceding experiment it was decided that no useful purpose would be served by pursuing the original plan of testing the remainder of the vaccinated

animals for immunity after a period of twelve months. In the light of the results which had been obtained by revaccinating cattle by the intravenous route it seemed possible, however, that the immunity might be reinforced and consolidated by a second inoculation, and that the experiment might, therefore, be profitably modified with this object in view. Accordingly, the second group of calves which had already been injected with an initial dose of 5 mg. or 50 mg. received

TABLE I

No. of Calf.	*Date of Vaccination.*	*Vaccine.*	*Date of Test Dose.*	*Duration of Life after Test Dose, in Days.*	*Post-mortem Findings.*
406	1–5–36	5 mg. BCG in olive oil	14–10–36	K. 177	Lesions in head glands. Moderate T.B. mesenteric glands. No. G.T.
414	1–5–36	Ditto	14–10–36	K. 183	Severe T.B. mesenteric glands. No G.T.
427	1–5–36	Ditto	14–10–36	K. 175	Moderate T.B. mesenteric glands. No G.T.
385	1–5–36	50 mg. BCG in olive oil	14–10–36	K. 188	One mesenteric gland, caseo-fibrous lesion. No G.T.
402	1–5–36	Ditto	14–10–36	K. 180	One mesenteric gland moderately infected. No G.T.
420	1–5–36	Ditto	14–10–36	K . 182	Lesion in a head gland. Slight T.B. mesenteric glands
428	1–5–36	Ditto	14–10–36	K. 174	One caseous colic gland. No G.T.
390 (Control)	—	—	14–10–36	K. 184	Severe T.B. of mesenteric glands. No G.T.
407 (Control)	—	—	14–10–36	K. 173	Lesions in head glands. Severe T.B. of mesenteric glands. Commencing G.T.

G.T. = Generalised tuberculosis.
K. = Killed.

a second and larger dose of the vaccine in the subcutaneous tissue of the brisket on the opposite side of the body. It was hoped that by this means the resistance of the animals might be appreciably raised.

Vaccination.—On May 1st, 1936, four calves (389, 392, 440, 443) received 5 mg. of BCG in gum acacia and olive oil as in the previous experiment, while 391, 394, 435 and 441 were injected with 50 mg. in a similar manner. Approximately twelve months later, *i.e.*, on May 6th, 1937, they were reinoculated with larger amounts of BCG also suspended in the oily substrate : five of the calves (389, 391, 392,

394 and 440) received 100 mg., while three (435, 441 and 443) were given 200 mg.

One calf (391) died from acute tympanitis on May 17th, 1937, before receiving the test dose. At autopsy, there was a firm fibrous swelling about 5 cm. in diameter in the brisket containing small pockets of caseo-pus from which a culture of BCG was obtained. There were no lesions elsewhere in the body.

A second calf (435) died from traumatic pericarditis and pleurisy on September 7th, 1937. The local lesion took the form of a small fibrous nodule the size of a bean in which no acid-fast bacilli were found. There were no lesions of tuberculosis.

Local Reactions in Vaccinated Calves.—In all but one calf the local lesions followed approximately the same course. Shortly after inoculation dense, bosselated swellings appeared in the subcutaneous tissue varying from 3 cm. up to 7 cm. in diameter. They increased in size for about eight weeks and then remained more or less stationary. Apart from one animal (392) in which the swelling ruptured at the eighth month liberating a thick caseo-pus, there was no tendency to fistula formation.

The only animal which reacted exceptionally was No. 443, which received a dose of 200 mg. Shortly after injection a deep-seated fibrous swelling not larger than a bean was detected in the subcutaneous tissue, but by the fourth month it had entirely disappeared and no trace of the injection could be found at autopsy. In the other animals well marked lesions were found at *post-mortem* examination. They varied slightly in extent, but were similar in appearance, being composed of granulomatous or fibrous capsules enclosing pockets of caseo-purulent material. In each case acid-fast bacilli were found in smears and in the one instance in which the pus was seeded on to suitable media a culture of BCG was recovered.

Tuberculin tests.—The results of serial tuberculin tests, applied at regular intervals after the inoculation of the 5 mg. and 50 mg. doses of BCG, have been described in the previous section. At the time the calves received the reinforcing dose of vaccine, *i.e.*, twelve months after the first inoculation, they were in a low stage of allergy. As might have been anticipated, the second injection resulted in a sharp rise in skin sensitivity which persisted at a high level until the termination of the experiment.

Immunity test.—On October 27th, 1937, the six vaccinated animals together with the three controls (403, 419 and 446) were subjected to an immunity test by feeding 5 mg. of virulent cultures of bovine tubercle bacilli as in the previous series. These three calves had been tuberculin tested on three occasions with negative results, but No. 419 was in poor condition. Shortly after the administration of the test dose this latter animal became ill and eventually died on November 17th, 1937. It showed very extensive abscess formations in the liver which were not of a tuberculous nature but were probably due to a foreign body.

Control Animals.

Calf No. 403 was killed on May 13th, 1938, *i.e.*, 198 days after the test dose.

Post-mortem findings.—Nine mesenteric glands showed caseo-calcareous lesions varying in size from 0·5 cm. to 1 cm. There were ten foci in all. There were similar changes in three colic glands. No visible lesions were found elsewhere in the body.

Two guinea-pigs were inoculated with a suspension from the affected mesenteric glands. One was found dead at the 19th day and showed numerous acid-fast organisms in a local gland. The other, killed at the 49th day, was affected with generalised lesions.

Calf No. 436 was killed on May 3rd, 1938, *i.e.*, 188 days after the test dose.

Post-mortem findings.—Two mesenteric glands were much increased in size and largely replaced by caseo-calcareous material, while millet seed sized lesions were found in three other glands. Two glands of the cæco-colic group were severely affected while one showed a single lesion. An anterior mediastinal gland was slightly enlarged and about one-half of its substance replaced by caseo-calcareous material. There was a pin-point lesion in the right iliac gland.

Two guinea-pigs were inoculated with a suspension from the affected mesenteric glands. Both were killed at the 48th day and showed generalised tuberculosis.

Vaccinated Animals.

Three calves (392, 440 and 441) were entirely free from visible lesions. In two animals (389 and 394) tuberculous changes, which were slight to moderate in extent, were seen in the mesenteric glands, while one calf (No. 443) was as severely affected as the controls since, in addition to lesions in the pharyngeal, mesenteric and cæco-colic glands, there was a tuberculous focus in a bronchial gland. (Table II.)

In the case of 392, 440 and 441, groups of guinea-pigs inoculated with pooled suspensions of various lymphatic glands, died or were killed at periods varying from 80 to 185 days. In each instance, there was no evidence of tuberculosis. Suspensions of affected glands from 389, 394 and 443 were also injected into guinea-pigs and produced generalised tuberculosis.

The extent of the lesions seemed to bear no relationship to the size of either the first or second vaccinating dose ; for example, No. 443, which was fairly severely affected had received the larger reinforcing dose of 200 mg. It is perhaps of some significance that this was the only animal in which the local reaction to the vaccinating dose was slight and in which no trace of a local lesion was detected at autopsy. In fact, there seemed to be some indication that the more severe the local reaction the greater the protection which was afforded by the vaccine.

TABLE II

No. of Calf.	*Date of First Vaccination.*	*Vaccine.*	*Date of Second Vaccination.*	*Vaccine.*	*Date of Test Dose.*	*Duration of Life after Test Dose.*	*Post-mortem Findings.*
389	1–5–36	5 mg. BCG in olive oil	6–5–37	100 mg. BCG in olive oil	27–10–37	K. 187 D.	Slight to moderate lesions mesenteric glands ; five affected. No G.T.
392	1–5–36	Ditto	6–5–37	Ditto	27–10–37	K. 196 D.	No lesions
440	1–5–36	Ditto	6–5–37	Ditto	27–10–37	K. 198 D.	No lesions
443	1–5–36	Ditto	6–5–37	200 mg. BCG in olive oil	27–10–37	K. 203 D.	Left pharyngeal gland, one mesenteric, two cæco-colic. Commencing G.T. (bronchial gland)
394	1–5–36	50 mg. BCG in olive oil	6–5–37	100 mg. BCG in olive oil	27–10–37	K. 189 D.	Moderate lesions, mesenteric glands. No G.T.
441	1–5–36	Ditto	6–5–37	200 mg. BCG in olive oil	27–10–37	K. 203 D.	No lesions
403 (Control)	—	—	—	—	27–10–37	K. 198 D.	Moderate lesions mesenteric glands ; nine affected. Three colic glands. No G.T.
436 (Control)	—	—	—	—	27–10–37	K. 188 D.	Moderate lesions mesenteric glands. Commencing G.T. (mediastinal and iliac glands)

Discussion.

Since Calmette and Guèrin published their initial series of articles on BCG numerous experiments have been undertaken on the vaccination of cattle by the subcutaneous route. The results of several such investigations were analysed in a previous article (Buxton, Glover and Griffith, 1934) and attention was drawn to the wide variations in the degree of immunity reported by different workers. It is scarcely possible to contrast these various experiments since differences in the age of cultures and the dose of vaccine, together with fluctuations in the interval elapsing between inoculation and complete resorption of the vaccine, may have influenced the final results, while the viability of BCG in the tissues is also subject to variations. It is highly probable that the majority of the organisms perish after a relatively short period, but living bacilli are sometimes recoverable from local lesions after a fairly lengthy interval.

With the object of delaying the absorption of the vaccine the incorporation of tubercle bacilli in an oily substrate has been advocated. Most of the experiments have been conducted in rabbits, guinea-pigs, etc. (Coulaud, 1935 ; Lagrange, 1935 ; Rist, 1937 ; Balteanu, Toma and Garaguli, 1937 ; etc.). Apart from the preliminary article which we published in 1934, no reference can be found to the protective value of similar suspensions in calves. In general, the available evidence suggests that the incorporation of the organisms in oil provides a ready method of inducing a state of allergy which is of a high order and is comparatively durable. On the other hand, there is little indication that the degree of immunity is any more substantial than that resulting from saline suspensions. In this connection, recent experiments of Balteanu, Toma and Garaguli which tend to confirm the fundamental observations of Rich (1933) and other workers are of some interest. These authors have noted some enhancement in immunity when guinea-pigs protected with BCG are rendered anergic by repeated doses of tuberculin, and they suggest that allergy is detrimental to immunity. As the calves used in our experiments were at all times sensitive to tuberculin it is conceivable that different results might have been obtained had they been desensitised before or after the infective dose.

In the case of the single injection of BCG in oil, the results of this experiment were less favourable than those obtained on the previous occasion. With the doses used, *viz.*, 5 mg. or 50 mg., a slight degree of protection was apparent in some of the animals, but the differences as compared with the controls was of little significance. The disappointing results on this occasion might be explained on the grounds that the amount of BCG injected was smaller or that the infective dose was more severe. Neither explanation, however, is entirely adequate, and it is considered more likely that the poorer results were due either to a less satisfactory response to the vaccine or to a higher sensitivity towards the test dose.

The series of calves receiving the double injection showed a

higher degree of resistance, but there were marked fluctuations as between different animals, which can probably be explained by variations in individual resistance or susceptibility. The test dose used was evidently adequate and was probably more severe than a natural infection.

Taken as a whole, the experiments give no indication that the subcutaneous inoculation of BCG in oil induces an immunity of sufficient solidity to warrant further experiments of this kind.

Conclusions.

(1) The subcutaneous inoculation of three calves with 5 mg. BCG incorporated in gum acacia and olive oil failed to induce an appreciable immunity to the oral administration of cultures of virulent tubercle bacilli six months later. When the dose of BCG was increased to 50 mg. a moderate protection was obtained in three calves out of four.

(2) A reinforcing inoculation of BCG given under the same conditions twelve months after a primary dose, induced a partial protection to the same infecting dose administered six months after the second injection of the vaccine. There were marked individual differences, however, since three calves out of six were completely protected, while in the remainder the lesions were almost as severe as in the controls.

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IMMUNITY EXPERIMENTS ON GUINEA-PIGS WITH KILLED TUBERCLE BACILLI AND BCG

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THE value of vaccination with inactivated tubercle bacilli has been the subject of much dispute and, as no agreement has yet been reached, the question is still worthy of further attention. In a recent article by Buxton and Glover (1934) it was shown that some immunity could be induced in calves by a single dose of tubercle bacilli killed by formalin and suspended in a mixture of gum acacia and olive oil or liquid paraffin. As the number of organisms used in these experiments was relatively great it was thought that smaller, repeated doses might give a better protection, and that bacilli killed in other ways might yield more potent antigens. It was realised, however, that an inquiry of this nature would demand a larger number of calves than was likely to be available.

The present investigation is concerned chiefly with the reactions of guinea-pigs to repeated doses of tubercle bacilli killed in various ways, and the subsequent immunity of the injected animals to a small test dose of virulent bacilli. It was hoped that by using a small infective dose in an animal which is highly susceptible to infection it might be possible to find out if there are differences in the immunising action of the different antigens. As a measure of control a group of guinea-pigs was also vaccinated with the BCG strain.

Many observers have demonstrated that some degree of immunity can be induced in the guinea-pig by the injection of dead tubercle bacilli, but the opinion formed, at any rate by earlier workers, was that the protection thus afforded was insufficient for practical purposes (Calmette, Guèrin and Breton, 1907 ; Romer, 1909, and others). Petroff and Stewart (1926) have pointed out, however, that in many of these experiments the infective dose was too massive to offer the most favourable conditions for the determination of a slight degree of immunity. Later workers have tended, therefore, to use small infective doses and, moreover, to give the animals a series of injections of vaccine instead of a single injection.

For example, Zinsser, Ward and Jennings (1925), Petroff and Stewart (1926), Petroff, Branch and Jennings (1929), have shown that guinea-pigs immunised with three or four doses of dead tubercle bacilli possess a slightly increased resistance to tuberculosis as compared with controls. Branch (with Cuff, 1930) claimed that guinea-pigs vaccinated intraperitoneally with 3 mg. of a heat-killed strain developed a definite degree of resistance to an intracutaneous injection of a small dose of virulent bacilli, but subsequently noted (with Enders, 1935) that better results are obtained by three intramuscular injections of organisms killed at 65° C.

Recently Lagrange (1935) has stressed the importance of multiple doses ofvaccine and has reported that, by inoculating guinea-pigs with several

doses of bacilli killed by warm formalin and continuing the injections after the administration of the infective dose, the protected animals survived at least ten months, whereas the controls died in four months. Pagel (1937) also induced a high degree of resistance in guinea-pigs by intraperitoneal injections (8 to 10) of heat-killed organisms with intervals of a week, the test for immunity being the intracutaneous inoculation of 0·001 or 0·01 mg. of a virulent human strain.

Experiments with living, avirulent organisms also have suggested the necessity for repeated injections before an appreciable degree of resistance can be induced in the guinea-pig and the favourable effect of numerous small doses of BCG has been recorded by Beereus (1933), Duprez (1934), Balteanu, Toma and Garaguli (1936). In one of the experiments of Schwabacher and Wilson (1937) there was " a slight suggestion that the animals which received ten doses of 0·1 mg. of BCG were better protected than those receiving one dose of 1 mg. The number of animals, however, was too small for the difference to be significant." Corper, Damerow and Cohn (1936) have shown that treatment of guinea-pigs with avirulent human or bovine tubercle bacilli given by the intracutaneous, subcutaneous or intravenous routes retarded the development of subsequent infection with virulent human or bovine bacilli, whereas equivalent amounts of heat-killed avirulent or virulent strains were without effect.

Present Investigation.

As previously mentioned, this investigation was made with the object of determining in guinea-pigs the relative immunising values of dead tubercle bacilli killed in various ways. The animals were given several doses of vaccine and were subsequently tested for immunity by injection into the cutaneous tissues or by instillation into the conjunctival sac of a small dose of virulent bovine bacilli.

Preparation of the Vaccines.—A bovine strain of full virulence was used in the preparation of the vaccines. Four amounts each of 200 mg. of young egg cultures were weighed out. Of these, three were suspended in salt solution and one in glycerin in the following way. A portion of each amount was taken and gently ground between roughened glass plates together with a drop of the diluent. The smooth paste thus produced was thinned with more diluent and then transferred from the plates to a test tube by pipette. A further portion of the culture-mass was taken and similarly treated, and so on until the whole amount was brought into suspension in from 2 to 3 c.c. of fluid. The four suspensions subsequently received different treatments. Two salt solution suspensions were heated in sealed glass tubes, one to 120° C. for 20 minutes, the other to 60° C. for 30 minutes. They were then diluted with normal salt solution in such proportion that the final suspension, amounting to 100 c.c., contained 2·0 mg. of culture per cubic centimetre. The third salt solution suspension was similarly diluted and treated with 0·5 per cent. formalin (commercial 40 per cent.) which was allowed to set on the bacteria for 24 hours at 37° C. The glycerin suspension was diluted with glycerin to make 20 c.c. and was then put in the incubator at 37° C. for nine days when it was removed and diluted with 80 c.c. of salt solution, the final suspension, therefore,

containing 2·0 mg. per c.c. Culture tests of the glycerin suspension at intervals during incubation showed that the bacilli were all killed within 24 hours. The glycerin vaccine was tested on a guinea-pig by subcutaneous and intraperitoneal injection. The animal was killed 24 days later and showed some peritoneal reaction but no sign of active tuberculosis and cultures from various regions remained sterile. The other vaccines were tested for sterility by sowing a few drops of the suspensions on to tubes of egg medium. The tubes remained sterile after several weeks' incubation.

Some of the suspensions though at first perfectly homogeneous underwent after a time a certain degree of auto-agglutination, the organisms forming compact clumps which were not dispersed completely by vigorous shaking. The clumping, most marked in the 120° C. suspension, may have led to some inequality in dosage as between individual animals and on different occasions but is unlikely to have influenced the final result.

The organisms treated with glycerin were not affected in this way and when shaken up produced a uniform suspension which was slow to sediment.

In addition to the suspension of dead organisms, a BCG vaccine was used for comparison with the dead vaccines. This contained 2·0 mg. of bacilli per c.c. suspended in normal salt solution and was freshly made for each vaccination.

Tuberculin Test.—Commencing on April 27th, and continuing until August 10th, 1937, a period of 15 weeks, intradermal tuberculin tests were applied at irregular intervals varying from one to four weeks, using a concentrated, precipitated, synthetic medium tuberculin. In the earlier stages a single dose of 0·1 c.c. of a 1:10 dilution in broth was injected into the skin of the abdomen, but later other dilutions, such as 1:100 and/or 1:500, were included. As it was not possible to test all the guinea-pigs on every occasion, an equal number (2 to 6), chosen at random, was used from each group. Care was taken that when a guinea-pig had been used for this purpose it was not included in the next test of the series.

Considerable differences in the intensity and extent of the reactions were noted as between individual animals, whereas the various groups, taken as a whole, tended to give more or less the same response on each occasion. It is thus impossible to draw any comparisons other than those of a general nature.

At the first test, applied on the 21st day, a 1:10 dilution induced a hæmorrhagic reaction with a necrotic centre in both the BCG animals which were tested and in one guinea-pig in the 60° C. vaccine group ; the remainder, seven in number, showed a definite thickening of the skin without hæmorrhage. Subsequently, the reactions were relatively mild and were far less intense than those which occur in guinea-pigs receiving a single dose of BCG or of a virulent strain of tubercle bacillus. This result was not altogether surprising as it is known that the repeated inoculation of a vaccine tends to desensitize

an animal to some extent. In general, the reactions took the form of a slight, moderate or marked œdematous thickening of the skin at the point of the injection. In about 15 per cent. of the cases the centre of reaction showed a hæmorrhagic infiltration, while in two instances only, apart from the first test, the 1 : 10 dilution induced slight necrosis. A 1:100 dilution, applied on the 105th, 112th and 125th days respectively, induced a slight or marked thickening of the skin, while a 1:500 dilution at the 125th day produced a slight reaction

Tuberculin Reactions.

Days.	Total	1/10				1/100				1/500			
		N	S	M	E	N	S	M	E	N	S	M	E
21	10	-	6	1	1H 2N	0	0	0	0	0	0	0	0
28	20	6	1 13H	-	-	20	-	-	-	0	0	0	0
38	12	-	1	6	3 2N	0	0	0	0	0	0	0	0
56	10	2	6	2	-	0	0	0	0	0	0	0	0
64	10	-	4	3	3	0	0	0	0	0	0	0	0
71	10	1	4	2	3	0	0	0	0	0	0	0	0
78	10	3	4	2	1	0	0	0	0	0	0	0	0
105	30	-	3	20 4H	3H	-	19	11	-	0	0	0	0
112	10	0	0	0	0	1	9	-	-	9	1	-	-
125	15	0	0	0	0	-	11	1 3H	-	9	6	-	-

N = no reaction.
S = slight thickening of skin.
M = moderate " " "
E = marked " " "
H = haemorrhagic centre.
N = necrotic "
O = not done.

in six animals only out of 15. The results are shown in the accompanying table.

Particulars and Results of the Experiment.

For the purpose of the experiment 96 normal guinea-pigs of approximately the same age and weighing from 500 to 600 grammes were selected. A small proportion was taken at random and tested intracutaneously with 0·1 c.c. of a 1 : 10 dilution of concentrated synthetic medium tuberculin. In each instance the test was negative

The animals were then divided into six groups of 16, five for vaccination each with a different vaccine, and one to serve as controls.

The groups were kept in separate cages and were housed in buildings which contained no tuberculous animals.

Between April 6th and June 23rd, 1937, a period of eleven weeks, nine injections of vaccine were given, the first three at weekly, the next three at fortnightly and the last three at weekly intervals. The first four doses were injected intraperitoneally. Before the fifth dose was due several animals had died and many had lost condition. The autopsies showed that the deaths were caused by the severe peritoneal reactions. The serous membranes were covered with fibrinous deposits and showed local areas of inflammation, particularly in the neighbourhood of the needle track, where adhesions of portions of the bowel to the parietal peritoneum were not uncommon. The intestines themselves were frequently matted together and adherent to one or more structures in the abdominal cavity, for example, the omentum, peritoneum, spleen and liver. Severe reactions occurred among the guinea-pigs inoculated with BCG and the heat-killed and formalinised bacilli and no fewer than 18 in these groups died during the course of vaccination, the deaths beginning after the third and occurring at intervals until after the last injection. It is interesting that none of the guinea-pigs which received the glycerin-vaccine died. The less severe reactions following this vaccine may be ascribed to the homogeneity of the suspension as compared with those of the other killed vaccines.

As it appeared probable that further intraperitoneal injections might cause a high mortality among the vaccinated animals, we decided to change the method for the subcutaneous route. After the fourth dose, therefore, the vaccine was injected subcutaneously in the flank on alternate sides. The subcutaneous injections gave rise to considerable local reactions but there were no deaths attributable to this method of injection, the deaths occurring after the change of method being due to peritoneal adhesions caused by the previous intraperitoneal injections. Twenty-four hours after the first subcutaneous dose of vaccine the sites of injection were examined for reactions. The findings are set out in the following table :—

Vaccine.	*No Reaction.*	*Slight.*	*Moderate.*	*Marked.*
			24 Hours.	
BCG ...	10	0	3	1
120° C. ...	2	5	4	3 (1 hæmorrhagic)
60° C. ...	1	5	8 (4 hæmorrhagic)	1 (hæmorrhagic)
Formalin	2	2	7	2
Glycerin	3	2	8 (2 slightly hæmorrhagic)	3 (2 hæmorrhagic)

The subcutaneous swellings slowly subsided and became abscesses which either ulcerated and discharged their contents or remained until the animal died or was killed. From one to four abscesses

or 45 altogether were found in 20 guinea-pigs on *post-mortem* examination. Of the 20 guinea-pigs five were in the 60° C., five in the formalinised, four in the glycerin, four in the BCG and two in the 120° C. group. Cultures were made from three abscesses in a guinea-pig which was killed 219 days after the last dose of BCG. The cultures remained sterile though acid-fast bacilli were readily found in the pus.

Fifty-one days after the last vaccination the survivors in each of the five vaccinal groups were divided into two sub-groups and all the animals in one set of sub-groups were tested by intradermal injection, and all those in the other set were tested by conjunctival instillation of fully virulent bovine bacilli. For each method of test there were eight controls. The same suspension of culture was used for all the guinea-pigs, the dose for each being 0·001 mg. of culture of the strain No. 34 B.V. It was intended to use for the resistance tests the same strain of bovine bacilli as was used in the preparation of the killed vaccines, namely, No. 28 B.V., but the suspension of this strain which was prepared for the purpose was not sufficiently fine—owing to the appearance of flocculi—for accurate dosage and was rejected. The dose for intradermal injection was contained in 0·1 c.c. and that for eye-instillation in 0·01 c.c. of physiological saline. The guinea-pigs were allowed to die naturally until the 23rd week, after which those still alive were killed. The direct cause of death of some of the animals was peritoneal adhesions, resulting from the vaccines.

Results in the Guinea-pigs Tested by Intradermal Injection.

Clinical.—By the 5th week all the controls, all the animals in the four groups vaccinated with killed bacilli and three of the six in the BCG group had distinctly enlarged inguinal glands, the degree of enlargement being greatest in the controls and least in one of the glycerin and in the three BCG animals; in the remaining three BCG animals the glands were not palpably enlarged. At the end of the 7th week all the BCG animals showed some degree of enlargement of the inguinal glands from slight to medium.

Post-mortem Findings.

Control Group.—Of the eight animals, one died prematurely in 26 days and showed besides an ulcer and caseous inguinal glands early lesions in an iliac gland and the portal gland and three tubercles in the spleen. The remaining seven died in from 81 to 101 days (average 92·2 days) of severe generalised tuberculosis, the spleen, liver and lungs in each instance showing extensive lesions and most of the glands some degree of caseation, the inguinal, iliac, portal and bronchial glands being caseous throughout.

BCG Group (six animals).—One died in 82 days of moderately severe, and two died in 118 and 120 days of moderate general tuberculosis. Two died in 123 and 124 days and showed caseation

of some glands, chiefly local, and only slight disseminated disease, one having one tubercle in the spleen and three tubercles in the liver, the other rather more tubercles in the liver and spleen and two dozen in the lungs. The remaining guinea-pig was killed after 165 days and showed a small local ulcer, large caseous inguinal glands, fibroid iliac glands with cavities containing thin pus and one small nodule in the portal glands ; other organs and glands were free.

120° *C. Vaccine Group* (six animals).—One died in 90 days of moderately severe general tuberculosis. One died in 92 days and the only glands showing enlargement and caseation were those adjacent to the seat of injection ; the spleen showed half-a-dozen foci on the surface and the liver and lungs no definite foci. The remaining four were killed 165 days after the test dose. One had general tuberculosis of moderate severity but showed less caseation and necrosis than the controls. Three had a chronic not severe form of general tuberculosis, the glands except the nearest being fibroid and translucent with few and small caseous areas, and the organs containing few nodules or tubercles.

60° *C. Group* (six animals).—One died in 93 days of non-tuberculous pneumonia ; tuberculosis was generalised but only moderate in liver and spleen and slight in lungs. Two died in 142 and 149 days ; there was extensive glandular tuberculosis but the visceral disease was moderate and not equal to that in the controls. One died in 134 days and showed extensive caseation of the different local, portal and bronchial glands but there were few tubercles or nodules in the spleen (five), liver (one) and lungs (two). Two were killed 164 days after the test dose ; one had widespread glandular tuberculosis (neck and axillary glands excepted) and slight visceral tuberculosis (liver scattered tubercles, spleen one softened nodule and lungs 24 or more ranging from 1 to 3 or 4 mm.) ; the other showed involvement of the usual glands—an inguinal and a xiphisternal having ulcerated through the skin—but most of them were dense and translucent with occasional caseous foci and there was only slight visceral tuberculosis (spleen twelve grey nodules, liver four caseous tubercles and lungs two or three grey tubercles).

Formalin Group (six animals).—One died in 85 days of volvulus and showed only slight visceral tuberculosis. Four died in 81, 108, 108 and 153 days of moderate to moderately severe G.T. The remaining animal was killed 164 days after the test dose and showed the usual glandular tuberculosis but with retrogressive features and only five grey nodules in the spleen and two grey tubercles in the liver.

Glycerin Group (eight animals).—One died in 39 days of severe G.T. (possibly the result of direct entry of bacilli into the blood stream at moment of injection). One died in 59 days from constricted bowel and showed very slight G.T. Three died in 116, 118 and 118 days of moderate G.T. Two died in 104 and 126 days respectively, and in these the usual glands were caseous but visceral lesions were

few. The remaining guinea-pig was killed 165 days after the test dose and showed caseation of all glands except neck and mesenteric but only five nodules in the spleen and two in the liver and sparsely scattered grey tubercles in the lungs.

Results in the Guinea-pigs Tested by Instillation into the Right Conjunctival Sac.

A month after instillation glands on the right side of the neck were palpably enlarged in six of the eight controls and a week later a gland was felt at the angle of the jaw in a further guinea-pig.

The remaining control never developed a palpable gland in the neck and did not react to tuberculin when tested 67 days after conjunctival instillation. Eighteen days after the tuberculin test enlarged glands were felt in the left inguinal region and eleven days later the animal began to breathe rapidly and was killed.

Autopsy.—The left inguinal glands were enlarged and caseating, the order of enlargement and degree of caseation being the reverse of what is found in cases of inoculation tuberculosis when the material is injected in the subcutaneous tissues of the abdomen. In this animal the two dorsally situated glands of the group were much enlarged, caseous throughout and softened, whilst that nearest the mid-line of the abdomen was only slightly enlarged and hyperplasic. This distribution led to search being made for a local lesion in the back which was rewarded by the finding of a small opening in the skin over the iliac crest communicating with a small subcutaneous collection of caseo-pus. Left iliac gland enlarged and caseous. Left axillary, a little enlarged. Spleen, two tubercles. A gland near right angle of jaw lying on carotid vessels, 5 mm. in diameter, was caseous throughout and partly softened. Lungs in a state of red hepatisation, not tuberculous. We think that this guinea-pig probably escaped infection from the test dose and became infected subsequently through a bite from one of its companions.

No glands were felt in any of the vaccinated guinea-pigs until the end of the 5th week when some degree of glandular enlargement, from just palpable to the size of a small pea, was detected in all the glycerin-vaccine group, all the formol group except one which was doubtful, four of the 120° C. group and two of the 60° C. group ; all in the BCG group were negative except one which was doubtful. At the 7th week the doubtful animal in the formalin group and the negative one in the 120° C. group each had a small indurated gland in the right submaxillary region ; two of the guinea-pigs in the 60° C. group, negative at the 5th week, now had slightly enlarged glands whilst of the BCG group one (not the one already mentioned) had a pea-sized gland and the rest were doubtful or negative. At the 9th week all those in the different vaccinated groups, which were doubtful or negative at the 7th week, had palpably enlarged glands, mainly slight in degree.

Post-mortem Findings.

Controls.—Excluding the guinea-pig which was apparently not infected by the instilled bacilli there are seven controls which died and showed on *post-mortem* examination the most advanced caseation in the glands of the neck. Two of the guinea-pigs died in 69 and 72 days of pneumonia and volvulus respectively, the tuberculous disease not being extensive. The remaining five guinea-pigs died of uncomplicated tuberculosis in from 72 to 143 days (average 102 days), the lesions being of moderate severity, none showing the very severe disease found in some of the intradermally inoculated animals.

After conjunctival instillation the lymphatic glands which show the most extensive and advanced caseation are the gland at the angle of the jaw on the side of the instillation and the mid-cervical glands on each side. Other glands in the neck are generally involved but to a less degree, those on the side of instillation to the greater extent.

Some of the guinea-pigs (control and vaccinated) showed lachrymation, thickening of the eyelids and congestion of the conjunctiva of the instilled eye, and also disease of the nasal cavity on that side.

BCG Vaccine Group (five animals).—Three died in 110, 117 and 168 days respectively, and two were killed 168 days after the test dose. Thus, the duration of life of three of the animals greatly exceeded that of the longest-lived control. In each instance the mid-cervical glands were caseous throughout but other cervical glands were not affected to the same extent as in the controls and the internal glands—thoracic and abdominal—when tuberculous were generally dense, translucent and fibroid with little caseation. The greatest difference, however, was in the amount of disseminated disease in the organs ; this was much less than in the controls, the spleens containing from 6 to 18 caseating tubercles or nodules, the livers scattered grey foci or tubercles and the lungs from 2 to 18 miliary tubercles.

120° *C. Vaccine Group* (five animals).—Four died in from 102 to 128 days (one from volvulus) and one was killed after 167 days. The last had moderately severe general tuberculosis. Three had only moderate disease, less than in the controls. The remaining one died in 126 days and showed slight disseminated tuberculosis, not obviously progressive.

Glycerin Vaccine Group (eight animals).—One died nine days after the test dose. Of the seven animals that remained three died in 89, 90 and 94 days and three in 134, 152 and 162 days and one was killed 167 days after the test. The three which died within 94 days showed the usual neck glands tuberculous and very slight disseminated disease ; in one the only organ affected was the spleen ; in another the liver and spleen contained a few tubercles only and in the third the spleen contained a nodule and a few tubercles and the liver and lungs each one tubercle. Of the remaining four guinea-pigs two had moderate and two had moderately severe general tuberculosis.

60° *C. Vaccine Group* (six animals).—One died in 72 days from peritoneal adhesions ; both mid-cervical glands were caseous

throughout and two others on the right side were less affected ; there was nothing internally except one minute tubercle in the spleen and one in the lungs. Four died in 100, 120, 146 and 149 days and one was killed 167 days after instillation. Two had moderate and three slight disseminated tuberculosis.

Formalin Vaccine Group (six animals).—These died in from 87 to 124 days (average 110 days). One had moderately severe general tuberculosis rather more severe than in any of the controls ; four had moderate general tuberculosis, on the whole rather less than in the controls, and one had slight disseminated disease similar to that found in the BCG animals.

Summary.

Intradermal Test.—Of the eight controls, one died prematurely and seven died in from 81 to 101 days of generalised tuberculosis which varied little in severity and extent of distribution, and was most severe in the animals which lived the longest.

The vaccinated animals, on the other hand, showed wide variations both in duration of life after the test dose and in the extent and severity of the lesions found at autopsy. Only eight guinea-pigs died within the control period and in three of these tuberculosis was not the immediate cause of death. Fifteen died of tuberculosis in from 104 to 153 days (average 124 days) and nine were killed in 164–5 days.

All the vaccinated guinea-pigs showed at autopsy local ulcers and enlarged uniformly caseous inguinal glands and in respect of local disease differed little from the controls. But in extent and severity of generalisation of the tuberculosis the guinea-pigs showed wide differences and on this basis are divisible into three groups. (*a*) Those with generalised disease severe in lungs, liver and spleen and thoracic and abdominal glands. (*b*) Those with generalised disease of moderate extent. (*c*) Those with generalised lesions few in number in the organs and fibro-hyperplasia in the lymph glands.

In the following table the numbers of guinea-pigs in each group are given.

Extent of Generalisation.

Vaccine.	*Moderately Severe.*	*Moderate.*	*Slight Visceral.*
BCG	1	2	3
Glycerin	1	3	4
120° C.	1	1	4
60° C.	–	3	3
Formol	2	2	2
	5	11	16

It will be seen that 50 per cent. of the guinea-pigs showed only slight visceral tuberculosis and that about two-thirds of the remainder fell into the intermediate group in which most of the animals lived

longer than any of the controls. It will also be observed that each of the vaccines increased the resistance of a proportion, varying from one-third to two-thirds, of the guinea-pigs in that group. The best results accrued from the bacilli killed at 120° C., followed closely by BCG. In the BCG group one guinea-pig showed a higher degree of resistance than any other vaccinated animal, the disease being limited practically to the seat of injection and the adjacent glands. The least good results were given by the formol vaccine and there was not much to choose between the glycerin and the 60° C. group.

Conjunctival Test.—One of the controls apparently escaped infection by the conjunctival route and became infected later probably by the bite of a companion. All the rest developed cervical gland and general tuberculosis from which five died in from 72 to 143 days (average 102 days), the lesions being of moderate severity. The two remaining animals died in 69 and 72 days of pneumonia and volvulus respectively and in these tuberculous disease was not extensive.

All the vaccinated animals became infected, the primary lesions being in the glands of the neck. An analysis of the *post-mortem* findings shows that the proportion of vaccinated animals with slight visceral tuberculosis was the same as in those tested by intradermal injection, namely, 50 per cent. But on comparing the different vaccinal groups one finds that the BCG vaccinated guinea-pigs exhibited the highest degree of resistance, all the animals, five in number, having less severe disease than the controls, three surviving the longest-lived control by 25 days, one of them being in good health when killed.

Of the animals treated with dead vaccines those which received the bacilli killed at 60° C. had the highest proportion, namely, two-thirds, of instances of slight disseminated tuberculosis. In the glycerin vaccine series there was distinct retardation of the tuberculous process following the test dose, but the final result was generalised tuberculosis not different from that found in the controls. The results in the 120° C. and the formalin groups were very similar ; in each group there was one animal with slight disseminated disease and one with moderately severe disease ; the rest had generalised tuberculosis rather less severe than in the controls, most of them dying within the control period.

Conclusions.

Each of the five vaccines—four of virulent tubercle bacilli killed by heat (60° C. and 100° C.) formalin and glycerin respectively, and one of BCG—raised the resistance of the great majority of the guinea-pigs to infection with virulent bovine bacilli.

The increase of resistance was not of a high order and was effective in diminishing the extent and severity of the generalised tuberculosis in only 50 per cent. of the guinea-pigs.

In about two-thirds of the remainder the vaccine exercised only a slight retarding influence on the progress of the tuberculosis and in the rest none at all.

On the whole the best results were given by BCG, one animal in this group showing lesions which were confined practically to the seat of injection and the adjacent glands, but there was little to choose between the results with the living vaccine and some of the dead vaccines.

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INVESTIGATIONS ON JOHNE'S DISEASE OF SHEEP

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During the spring of 1932, Johne's disease was diagnosed in a flock of over 300 Southdown ewes with lambs at foot. Since the beginning of the year a few deaths had occurred among the ewes but the cause had not been investigated; from the reports received, however, regarding these cases it is probable that the animals had died from Johne's disease.

During the nine months commencing April, 1932, 26 ewes selected by the shepherd as diseased were received at the laboratory and examined; 16 of these animals were received before the end of June. The majority were found infected with Johne's disease, but although all showed symptoms of the disease the infection was not demonstrated in every instance. In the same period a few sheep died at the farm but were not examined.

Throughout the two following years cases of Johne's disease continued to occur in the ewe flock but the losses were not so heavy as in 1932. Nevertheless the farmer gave up breeding from the ewes of this flock and the animals were prepared for slaughter. The disease was still recurring in 1935 before the animals were disposed of, and it was diagnosed in four non-pregnant ewes sent to the laboratory in January of that year.

Clinical cases occurred at all times of the year but the majority were found when the ewes were suckling their lambs. The occurrence of active cases at this time was no doubt responsible for the infection of some of the lambs and it was probably responsible for the successful perpetuation of the infection in the flock which was self-contained on the ewe side. Throughout the rearing season the ewes and lambs grazed over the South Downs during the day and at night they were folded on arable crops. The fæces from infected ewes were generally soft in consistency, sometimes diarrhœic and usually heavily contaminated with Johne bacilli. These contaminated the limited area of crops on which the animals were folded each evening, and this must have greatly increased the chances of the lambs from healthy ewes ingesting considerable numbers of the bacilli. The lambs of infected ewes suffering from a diarrhœa that soiled the wool of the udder must have been exposed to the risks of a heavy infection even when not on the fold.

It was not possible to trace the introduction of infection into this flock and, although young cattle grazed over the same Downs as the sheep, they were healthy home-bred stock and a clinical case of Johne's disease among the older animals was said to have been unknown for several years.

The ewe flock was tested with johnin in May, 1932. Prior to the flock test a preliminary test on three infected ewes and six normal sheep had been made at the laboratory when the animals were inoculated in the upper eyelid with 0·1 c.c. of johnin prepared from a bovine strain of the bacillus. The readings of this test were taken at 24 and 48 hours after inoculation. The three diseased sheep showed swelling of the inoculated eyelid with congestion of the conjunctiva and some injection of the vessels of the conjunctiva and sclera. The normal sheep showed no reaction whatsoever. The flock was tested in the same manner and 29 ewes gave a very strong reaction, the upper eyelid being markedly swollen, the eye closed or almost closed and the conjunctiva showed a correspondingly definite inflammatory response. Twenty-seven ewes gave a medium reaction and 26 a less marked reaction but even the latter showed definite swelling of the eyelid and congestion of the conjunctiva. The remainder of the ewes, 263 in number, showed no reaction. At the time this test was made seven ewes showed clinical symptoms suggestive of Johne's disease but only five of these reacted to the test.

A comparison of the age incidence of the reacting ewes and ewes shown to be diseased at *post-mortem* examination showed that this was approximately the same for both. After the johnin test a random sample of the flock showed that 36 two-toothed, 20 four-toothed and 14 six-toothed ewes had reacted to the test.

The records of the *post-mortem* examinations, carried out on 14 ewes a few weeks up to six months after they had been tested with johnin in the field, are given in Table I. Eleven of the animals were retested shortly before they were slaughtered for examination. The table shows that there is no constant agreement between the results of the test and the *post-mortem* findings.

Table I.

No. of Sheep.	*Result of Herd Test.*	*Result of Test before Post-mortem Examination.*	*Diagnosis of Johne's Disease at Post-mortem.*
1	—	—	+
2	+	+	—
3	—	—	+
4	+	+	+
5	+	+	+
6	+	+	+
7	+	+	—
8	—	—	+
9	—	—	+
10	+	—	—
11	+	—	+
12	+		+
13	+		—
14	+		—
15	+		—

It should, however, be noted that in sheep the macroscopic lesions may be insufficient to arouse a suspicion of the disease. Furthermore, a careful examination of smears made from the mucous membrane of the intestine at intervals of 18 inches throughout its length has in one instance failed to show any Johne or acid-fast bacteria, but sections of the intestinal wall showed the characteristic accumulations of epithelioid cells that result from an invasion of the mucosa with the Johne bacillus and although bacteria could not be demonstrated the microscopical appearance of the bowel justified a diagnosis of Johne's disease being given. In Table I the negative diagnosis was made in four instances without sections being examined and it is, therefore, possible that some of these animals may have been infected but not diagnosed as such.

The ewe teg flock of 137 animals was kept separate from the ewes but grazed over the same area of downland. This flock was tested with johnin in June, 1932. One gave a strong reaction, one showed a moderate reaction and 14 gave weak reactions. All of the animals in this flock were in beautiful condition and nothing further was done to the animals. They were, however, added to the ewe flock after lambing the following spring.

Besides the Southdown sheep there was a flock of 75 Cheviot ewes that had been on the farm for twelve months and a small flock of 24 Kerry Hill ewes that had been on the farm for three months only. Both of these small flocks though not coming into direct contact with the Southdown sheep grazed the same downland and at times may have grazed pasture run over some time previously by the Southdown ewe flock. A johnin test on the two small flocks showed nine reactors among the Cheviots and four among the Kerry Hills but none of these sheep had shown any signs of Johne's disease nor did the disease make its appearance in these flocks at a later date.

A further test was carried out on normal sheep belonging to the College where Johne's disease was unknown. The result was as follows :—

39 one- and two-lamb Kent ewes : all negative.

39 three- and four-lamb Kent ewes : five reacted.

32 two-lamb Border Leicester Cheviot cross-bred ewes : two reacted.

One of the Kent ewes gave a very strong reaction, the inoculated eyelid being very swollen. This ewe was killed and examined, but no evidence of Johne's or any other disease was found.

The results of these tests show : (*a*) In an infected flock a higher proportion of the animals react to johnin than in a flock where the disease is unknown. (*b*) The positive reactions shown by healthy sheep indicate that some non-specific sensitisation to the products of the Johne bacillus occurs and that this may be commoner in older animals. (*c*) That infected sheep sometimes show no reaction to the test.

It was, therefore, concluded that johnin prepared from the

bovine strain of the bacillus was of little value in the diagnosis of the disease in sheep.

Symptoms of the Disease in Sheep.

Naturally and experimentally infected sheep show similar symptoms. There is a loss of condition which is frequently very rapid, leading in a short time to extreme emaciation, cachexia and death. When loss of condition becomes very evident the fæces are soft and pultaceous and may even be liquid, but diarrhœa is by no means a constant symptom, the fæces being frequently no less formed than those of normal sheep on succulent pasture. In advanced cases, the visible mucous membranes are paler than normal. There is nothing in these symptoms to distinguish the disease from parasitic gastro-enteritis but in the later stages a diagnosis of Johne's disease is generally obtained by the demonstration of the acid-fast bacilli in characteristic clumps in the fæces. A condition frequently noted in the Southdown ewes but less evident in the experimental sheep which were of the Kent breed was the ease with which the fleece pulled out on handling the animals, leaving areas of pallid, greasy-looking skin. In parasitic worm infestation a comparable tendency to shed or lose the fleece on handling has not been observed. That this shedding of the fleece is not an uncommon condition was suggested by Stockman (1911), who mentioned a case of Johne's disease having been diagnosed in an animal which was supposed to have been suffering from scrapie for several months, and Dunkin and Balfour-Jones (1935) record the loss of fleece in cases of the disease ; furthermore, the picture of an affected sheep given by Howarth (1932) indicates that the fleece pulls out readily.

The majority of cases in the affected flock occurred in lactating ewes and at this time the disease tended to run an acute course, the animal dying three or four weeks after symptoms were first noted. Affected ewes have, however, been observed over periods of several months during which time some improvement in general strength occurred, but this has generally been followed by a more acute form of the disease.

Post-mortem Findings.

The carcases of sheep that had died or were slaughtered *in extremis*, were almost completely depleted of fat, but occasionally the appearance of the fat reservoirs in the abdominal cavity was peculiar in so far as the whitish firm fat was replaced by a gelatinous semi-translucent greyish yellow material interspersed with small, opaque whitish flecks, the latter being suggestive of residual deposits of the normal fat.

Some thickening of the walls of the intestine, particularly of the jejunum, ileum, cæcum and less frequently of the colon, was usually evident. This thickening was generally best seen in the ileum. The mucous membrane of the thickened parts sometimes showed

slight corrugation and a variable degree of hyperæmia. On stripping the small intestine from the mesentery transverse fissures of the thickened mucous membrane were sometimes produced and were found as gaping cracks on opening the bowel. Furthermore, on stripping, the thickened parts of the small intestine showed a greater tendency to break than does the normal intestine. The abomasum and duodenum were generally normal but in exceptional cases, of which only two were encountered, their mucous membranes were found markedly hyperæmic and microscopical examination showed a heavy infestation with Johne bacilli. The thickened areas of the intestine were just as readily detected in the unopened bowel *in situ* as when the bowel was slit open, and were recognised by their more opaque and solid appearance and by their thickened doughy feel. Furthermore, in the great majority of instances, the lymph vessels draining the thickened bowel presented a very characteristic appearance, these vessels standing out below the peritoneal covering over the bowel as distended opaque or semi-translucent cords running an irregular or slightly tortuous course towards the mesenteric border, with here and there along their course small whitish opaque and firm tubercles that had, as far as could be ascertained on careful sectioning, a solid consistency. On cutting across the distended lymphatic vessels their liquid contents escaped and the vessels collapsed and became much less prominent. The lymph vessels in their course from the mesenteric attachment of the bowel to the lymph glands were generally less conspicuous than over the bowel itself, but stood out as prominent opaque or semi-transluscent cords traversing a straight course through the mesentery now denuded of fat until lost by ramification in their respective glands.

The lymph glands draining the affected portions of the bowel were generally normal in appearance and consistency. Sometimes the mesenteric lymph glands appeared enlarged but the same glands in normal sheep show a corresponding range of diversity in size and little or no reliance can be placed on the impression of increased size. Enlargement of the smaller lymph glands lying adjacent to the colon was a feature more commonly recognisable and is probably of more diagnostic value than enlargement of the mesenteric lymph glands.

No other specific lesions were encountered but the organs were anæmic.

The Demonstration of the Bacteria.

The bacteria were generally found in very large numbers in smears made from the mucous membrane of the affected portion of the intestine. The jejunum, ileum, cæcum and colon were the most heavily infected portions of the bowel but in rare cases the bacteria have been demonstrated in large numbers in the mucous membrane from the abomasum to the rectum. The numbers of bacteria encountered in smears was generally very great indeed, sometimes

the red masses of bacteria being more prominent than the blue stained cells of the mucosa.

The bacteria were demonstrable in considerable numbers in the lymph glands draining heavily infected portions of the intestine but at times it has been impossible to demonstrate them in smears from the lymph glands draining portions of moderately infected intestine. The lymph glands draining tissues other than the infected areas of intestine have frequently been examined for bacteria but none has been found.

Histological Findings.

The lesions in sheep whether in the intestine or in the lymph glands are confined to accumulations of one type of cell, namely, the macrophage or large epithelioid cell, and their distribution follows almost exactly that of the bacilli which are phagocytosed by the cells and apparently multiply in them. Thus, when only a few bacilli are present at a particular site a small collection of epithelioid cells will be found but wherever the bacilli are abundant large numbers of the cells collect. No cellular reaction other than the accumulation of epithelioid cells occurs but the epithelioid cells crowd out and replace the cells normal to the part.

The accumulated epithelioid cells are conspicuous in the mucous membrane of the intestine and in advanced lesions they cause an almost complete disappearance of the normal cells between the glands from the base of the intestinal epithelium to the *muscularis mucosæ*. The *muscularis mucosæ* frequently acts as a barrier to the deeper penetration of the bacilli and the accompanying cells, but not infrequently the barrier is surmounted and the invasion of the submucous coat may be either slight or extensive. In the latter case the *muscularis mucosæ* itself may be partially or almost completely obliterated by the epithelioid cells which advance in massed formation into the reticular submucous tissue converting this to a more or less solid consistency. This extensive invasion has been noted particularly in the cæcum. In the small intestine the submucous invasion is less extensive and smaller collections, often isolated and consisting of a few cells only occur. Varying degrees of cell infiltration were found in the mucous membrane itself, but the surface epithelium and the tubular glands are always spared, though the latter may appear to undergo obliteration by excessive accumulations in the surrounding tissues. The lymph follicles show a high degree of resistance to invasion by bacilli and epithelioid cells may remain intact when the surrounding normal tissues have been all replaced, but even the lymph follicles occasionally show peripheral infiltration of bacilli and epithelioid cells. The epithelioid cells themselves have a large vesicular nucleus often placed eccentrically and an abundant cytoplasm that may contain a quite remarkable number of bacilli. In large accumulations of these cells the individuals lie close to one another and although the cell outline is not lost the appearance

of a syncytium as described by McFadyean (1918) is given. Giant cells have seldom been noted in intestinal lesions.

In the lymph glands the greatest number of bacteria and epithelioid cells are found in the cortical lymph sinuses but an irregular peripheral invasion of the lymph nodes occurs. An increase in the number of macrophages or epithelioid cells may be noted in the medullary sinuses and occasionally small collections of epithelioid cells are found in a medullary ray. Bacilli are demonstrable in some but not all of these sites of cell infiltration.

The enlarged lymph vessels have greatly thickened walls and may be either partially or completely obstructed by a mass of material consisting of moderate numbers of cells and fibrin. Some of the cells are epithelioid cells, the majority of which are in various stages of disintegration; moderate numbers of lymphocytes are also present. The centres of these masses often show extensive necrosis presenting a homogeneous ground-work through which are scattered fragmented nuclei and bacilli. A few epithelioid cells surrounded by lymphocytes may be found invading the walls of the lymph vessels and the deeper tissues and these cell collections are also found partially embedded in the tissues and partially protruding into the lumen of the vessel. The cell structures of these small collections may be intact, as may be that of comparable small cell groups found free in the lumen of the vessel. In the later stages the lumina of the lymph vessels are irregular and partially obliterated by the pressure of the proliferating fibroblasts in the now greatly thickened walls outside the muscular coat and finally the entire structure of the lymph vessel is lost having been replaced by a mass of fibrous tissue.

The complete absence of necrosis in the lesions in the mucous membrane and submucous coat of the intestine and in the lymph glands is in marked contrast to the necrosis of the cell accumulations in the obstructed lymph vessels. In the former sites the blood vessels are normal and the nutrition of the invading cells is maintained whereas in the obstructed lymphatics the nutrition is probably insufficient to maintain the vitality of the cells that have accumulated and the necrosis is particularly evident in the bacteria-laden epithelioid cells. The lesions in sheep are, as already noted by Twort and Ingram (1913) essentially similar to those found in cattle. McFadyean (1918) described accumulations of plasma cells in the mucous membrane and submucous coat and regarded these as the precursors of the epithelioid cells. In sheep an increase of plasma cells has not been recognised, but these cells together with lymphocytes and a number of eosinophiles are present in normal numbers in the mucous membrane until crowded out by the epithelioid cells. The giant cells which are well recognised in cattle have but rarely been found in sheep and this is in general agreement with the observation by Lange and Berge (1915) to the effect that giant cells were not found in the intestinal lesions of sheep. Hallman and Witter (1933) suggest that in cattle the extensive hyperplasia of the mucosa results in a

blocking of the lymphatics and of the blood vessels and that this may be a primary factor in the superficial necrosis of the mucous membrane which they sometimes found. In the present investigation necrosis of the mucous membrane has not been seen and the capillaries of the mucous membrane where cell infiltrations have been most extensive have been found intact and patent. A shedding of the intestinal epithelium must occur and with it some escape of underlying epithelioid cells and bacteria, otherwise the appearance of bacilli in characteristic clusters in the fæces would not occur, but whether the shedding of the mucous membrane is in excess of the physiological is not known. In sheep there is undoubtedly an obstruction to the free flow of lymph from the intestine but this occurs primarily in the obstructed or partially obstructed lymphatics outside the mucous membrane and in the lymph glands where the collections of epithelioid cells in the lymph sinuses will act as a barrier to the lymph flow.

The typical accumulations of epithelioid cells have been found in the intestine of an animal that had shown symptoms of the disease when bacilli were not found either in smears from the mucous membrane or in suitably stained sections. This suggested that a resistance or immunity to the Johne bacilli may sometimes be attained and the bacilli effectively disposed of, the epithelioid cell accumulations remaining as witness to the former activity of the infection.

Attempted Cultivation of the Bacteria.

Numerous attempts have been made to cultivate the bacteria on the media ordinarily used for the cultivation of the Johne bacillus and on various modifications of these media. The basis of the media was generally a liver broth, egg mixture modified by the addition of various proportions of glycerine and *Myco. phlei* or *Myco. tuberculosis* either avian or bovine. Seed material has consisted of massive numbers of bacteria from the intestinal mucous membrane treated with antiformin or NaOH in various concentrations and for different lengths of time. The material so treated has been sown out without subsequent washing and after washing with saline. The infected lymph glands from slaughtered animals have been removed with sterile precautions and material from them, rich in bacilli, inoculated on to various media, some with and some without the addition of gentian violet. This untreated seed material though sometimes contaminated has frequently been proved free from contaminating micro-organisms. The inoculated culture media has been incubated at 37° C. for periods of three, six and more months, but in no case has there been definite evidence of the growth of the Johne bacillus. It was, therefore, concluded that this ovine strain differed from the much more readily cultivated strains that are ordinarily recovered from cattle, and from the readily cultivated strains that have been isolated from sheep by some workers.

Infection Experiments.

A suspension containing very large numbers of the bacilli was obtained by grinding up scrapings from the infected intestinal

mucous membrane in saline and allowing the coarser particles to sediment. One cubic centimetre of this suspension was inoculated intravenously into each of five rabbits. Two of these rabbits died within three weeks of the inoculation but the three remaining rabbits were examined at *post-mortem* between five and six months after the inoculation and showed no evidence of any disease associated with infection with acid-fast bacilli.

A number of young guinea-pigs were fed daily with infected mucous membrane during their first two weeks of life but these animals showed no signs of any disease when they were killed and examined five to six months later.

Twenty cockerel chicks were fed when one day old with infected mucous membrane and on the four following days three to four drops of a suspension of the bacilli were given to each chick by the mouth. A number of these chicks were accidentally destroyed when they were eight weeks old but eight survived and they were examined when six months old and none showed any signs of disease.

In order to maintain the strain a number of young lambs have been fed infected bowel each year since 1934. The results of these experiments may be summarised by saying that the disease is produced with considerable regularity in animals fed as sucking lambs with large quantities of the bacteria in material from cases of Johne's disease. No adult sheep fed with similar material and kept in contact with sheep in the small experimental flock has become infected.

The results of the first two years' experiments on lambs will be sufficient to show the susceptibility of young animals to the infection.

In April, 1932, three young lambs were each fed daily for five days 5 grammes of infected mucous membrane from the intestine of a sheep. One of the lambs died on November 7th, 1932, and while showing evidence of parasitic gastro-enteritis at the *post-mortem* examination, Johne bacilli were found in clusters in every third or fourth microscopic field of smears made from the mucous membrane of the ileum, cæcum and colon. The second lamb was killed on December 30th, 1932, after showing symptoms of the disease for several weeks. There was no thickening of the intestinal mucosa or other macroscopic evidence of infection but Johne bacilli in very large numbers were found in the latter two-thirds of the small intestine and cæcum, and in small numbers in the glands draining these parts. The remaining lamb died on January 16th, 1933, and showed evidence of parasitic gastro-enteritis but Johne bacilli were found in small numbers in the ileum ; they were not found in other parts of the intestine nor in any of the lymph glands.

In the spring of 1934, eight ewes and nine lambs were purchased and all were fed with infected material at the end of June when the lambs were about ten to twelve weeks old. By January, 1935, only six of the lambs were alive, one had been drowned and two

others had died of parasitic worm infestations. In February, 1935, one lamb was killed *in extremis* and found heavily infected with Johne's disease. In April, another lamb died from the disease ; the third died in August, a fourth in October and the fifth in November, all from Johne's disease. The remaining lamb never showed any evidence of infection and was killed and found healthy at the end of 1938. From the 1932 and 1934 experiments on lambs, it is seen that eight out of nine animals that survived for six months or more after infective material was fed contracted the disease. This is in marked contrast to the completely negative results obtained by the feeding of adult sheep. There has been no opportunity to test the susceptibility of numbers of animals from the time of weaning until the adult stage but in 1937 two eight-months' old lambs were fed with mucous membrane from the intestine of an infected sheep, both of these lambs had been in contact with the experimental flock for two to three months before they were fed. These two lambs suffered from a permanent photosensitisation of the skin and had learned to avoid the light, remaining in a shed during the greater part of the day. They were, in consequence, rather under-nourished and poorly grown. One was a ram and the other a wether lamb, and both were in very poor condition twelve months after having been given the infected material, and were then slaughtered and examined and found to be extensively infected. It, therefore, appears that the susceptibility of lambs is continued beyond the weaning age and this is in agreement with the successful infection of yearling sheep obtained by McFadyean and Sheather (1916) who inoculated the animals intravenously with a culture of the bacillus.

The Infection of Calves.

It was considered advisable to try and ascertain whether this ovine strain of the bacillus was infectious for cattle and in 1935 six young heifer calves were fed with infected material from sheep. The amounts fed were purposely large so that had negative results been obtained the experimental exposure would have been sufficiently severe to justify the conclusion that the ovine strain was not infective for calves by the oral route. The strain proved infective for calves, all six animals becoming infected.

Calves Nos. 1, 2, 3 and 4 were given their first feed of infected material when a few days old, each receiving infected intestinal mucous membrane from sheep as follows : 15 grammes on January 24th, 25th and 26th, 1935 ; 5 grammes on February 20th, 1935 ; 20 grammes on April 9th, 1935 ; 20 grammes on June 20th, 1935 ; 20 grammes on July 11th, 1935 ; 10 grammes on October 10th, 1935.

Calves Nos. 5 and 6 were also first fed infected material when a few days old. They received infected intestinal mucous membrane from sheep as follows : 7 grammes on February 20th, 1935 ; 20 grammes on June 12th, 1935 ; 50 grammes on July 11th, 1935 ;

45 grammes on October 10th, 1935 ; 45 grammes on November 6th 1935.

The calves were tuberculin tested in July and November, 1935, and all gave negative reactions on both occasions. *Post-mortem* examinations have been made on five and none has been found infected with tuberculosis.

Calf No. 1 remained in normal health until March, 1936, but from that date until the end of June, when the animal was slaughtered in an emaciated state, loss of condition had been progressive. Slight diarrhœa was noticed for a few days during the middle of March, but during the two to three following weeks the fæces were normal. However, by April 6th, diarrhœa was persistent and continued with one short remission of a few days until the animal was slaughtered. From November, 1935, the fæces were examined at approximately weekly intervals for Johne bacilli, smears of the fæces being made and stained in the usual way. Bacilli were found in clumps on the following dates : December 24th, 1935 ; June 15th, 1936 ; June 22nd, 1936 ; and June 29th, 1936.

Calf No. 2.—The animal was in good condition and normal health until April, 1937, wheh she began to lose flesh and by May, she was suffering from diarrhœa and presenting the clinical appearance of a case of Johne's disease. The diarrhœa persisted throughout May and into June, when it stopped for two or three weeks and the general apcarance of the animal showed some improvement. Diarrhœa recommenced in July, but in August the fæces had recovered their normal consistency and this was maintained until the middle of November. On November 23rd, diarrhœa was evident and from then it persisted until the animal was slaughtered in an emaciated condition on December 12th, 1937. The bacilli were identified in the fæces at the weekly examinations on the following dates : clumps of bacilli on September 8th, 1936 ; small numbers of single bacilli but not clumps present on October 6th, 1936 ; October 13th, 1936 ; October 22nd, 1936 ; November 2nd, 1936 ; December 15th, 1936 ; January 25th, 1937 ; April 18th, 1937 ; May 20th, 1937 ; May 24th, 1937 ; June 7th, 1937 ; June 14th, 1937 ; clumps of bacilli were found on July 5th, 1937, and single bacilli on July 19th, 1937 ; clumps of bacilli again present on September 20th, 1937 ; October 4th, 1937, and October 11th, 1937 ; single bacilli present on October 18th, 1937 ; clumps of bacilli present on November 2nd, 1937, and November 29th, 1937 and single bacilli present on December 6th, 1937.

Calf No. 3.—This animal gave birth to a calf on March 30th, 1938, and suckled it. It remained in apparently normal health until the middle of September, 1938, when it began to lose condition steadily until it was slaughtered in a poor but not emaciated state on November 24th, 1938. She showed intermittent looseness of the bowels for six weeks before death but not profuse diarrhœa. During May, 1936, the fæces had been loose but this may have been due to the freshness of the new pasture.

The fæces were examined at weekly intervals from November, 1935, till May 31st, 1938. Regular weekly examinations were recommended on September 11th, 1938, and continued until the animal was slaughtered. The results of these examinations were as follows: Single bacilli in small numbers present on November 2nd, 1936; December 7th, 1936; January 25th, 1937; February 2nd, 1937; April 1st, 1937; April 5th, 1937, and November 2nd, 1937; clumps of bacilli present on November 21st, 1938.

Calf No. 4.—This animal gave birth to a calf on January 8th, 1938, and suckled it. The cow is still alive and at the time of writing, January, 1939, is in good clinical condition. In April, 1937, the animal lost condition rapidly and suffered from a profuse diarrhœa. She then showed the symptoms of Johne's disease but in May recovery commenced and by the middle of June, the animal was again in good condition.

Bacilli were recognised in the fæces on the following dates: Single bacilli were present on November 2nd, 1936; November 30th, 1936; clumps of bacilli were present on December 7th, 1936; single bacilli present on December 15th, 1936; clumps of bacilli present on December 20th, 1936 and November 5th, 1937; single bacilli present on January 25th, 1937; February 2nd, 1937 and March 8th, 1937; clumps present on April 1st, 1937 and April 5th, 1937; isolated bacilli present on November 2nd, 1937. Thereafter the fæces were negative at the weekly examinations up to May, 1938, when the examinations were stopped. Examinations were recommenced from September 11th, 1938, until the end of December but no bacilli were found.

From the clinical condition of the animal in April, 1937, and the frequent presence of Johne bacilli in the fæces either singly or in clumps it is concluded that this animal had passed through an active stage of Johne's disease.

Calf No. 5.—The typical symptoms of the disease asserted themselves in April, 1936, and persisted until the animal was slaughtered *in extremis* on July 9th, 1936. At the weekly examinations of the fæces bacilli were found on the following dates. Single bacilli present on March 10th, 1936; April 6th, 1936; clumps present on April 13th, 1936; single bacilli present on April 20th, 1936; May 25th, 1936; clumps of bacilli present on May 2nd, 1936; July 8th, 1936; June 22nd, 1936, and June 29th, 1936.

Calf No. 6.—The typical symptoms of Johne's disease were observed in December, 1936, emaciation was rapid and the animal had diarrhœa for several days in May, 1936, and again for a few days during the middle of September, 1936. During October, the fæces were softer than normal but not liquid in consistency. Bacilli were present in the fæces on the following dates: Single bacilli present on June 21st, 1936; clumps of bacilli present on August 31st, 1936 and September 8th, 1936; single bacilli present on October 12th, 1936; clumps of bacilli present on October 20th, 1936 and November

7th, 1936; single bacilli present on November 30th, 1936; clumps of bacilli present on December 7th, 1936 and December 15th, 1936; December 30th, 1936; January 5th, 1937; January 18th, 1937 and January 25th, 1937. Slaughtered *in extremis*, January 31st.

The *post-mortem* examinations on the five animals that were slaughtered were all typical for advanced cases of Johne's disease. Animal No. 1 showed a very heavy infection of the whole of the small intestine from within 2 feet of the pylorus and a heavy infection of the cæcum and colon and a moderate infection of the terminal part of the large bowel. No. 2 showed a heavy infection throughout the small intestine except for the first 15 feet which was not affected. The cæcum and first 8 feet of the colon were infected. No. 3 showed an infection similar in extent to No. 2. No. 5 showed a heavy infection similar to No. 1. No. 6 showed an infection similar in extent to No. 2. Attempts to cultivate the bacilli from animals Nos. 1, 3, 5 and 6 were unsuccessful and indicated that passage of the ovine strain through the bovine had not brought the strain culturally into line with bovine strains.

Johnin Tests on the Experimental Cattle.

The usual double intradermal test was carried out and the results are summarised in Table II. In this table, the approximate dates when diarrhœa was first noticed and bacilli were first recognised in the fæces are indicated.

As a control to these Johnin tests, ten young animals ranging from three months to eighteen months were tested in July, 1936. These came from a self-contained tuberculin tested herd where there has been no known case of Johne's disease for several years and where none has occurred since the test was made. The young stock were kept on separate premises from the cows. The results are shown in Table III.

Although No. 8 showed a reaction comparable to that given at times by some of the experimental cattle there is no reason to suppose that she was an infected heifer. The possibility of infection cannot, however, be completely ruled out. The other animals gave no appreciable reactions. From a comparison of the results obtained in the normal and infected cattle it would appear that an increase of more than 3 millimetres on the initial skin measurement is suggestive of a positive reaction.

The chief characteristic of the swellings obtained in the infected cattle was their firm, cool and painless character but this was not invariably the case as occasionally a painful and warm swelling was produced, but the cool and painless character of the swelling was so constant that in the majority of cases a positive or negative diagnosis had to be given on the size of the swelling only. An examination of the measurements will show that considerable variations were shown in the extent of the reactions produced in the same animal at different tests. In view of the results obtained with

TABLE II.

	Date.	Measurements. 0 Hours.	Or Type of Swelling at 48 Hours.	72 Hours.	Type of Swelling.	Reaction.
No. 1	2.7.35	8	14	24	F, WD	+
	12.12.35	8·5	S, Fl, F, C.	17·5	F, C, WD	+
Diarrhœa in Mar., 1936	9.3.36	8	12	11·5	S, WD	—
Bacilli in fæces, 24.12.35	4.5.36	7·5	12	16	F, WD	+
No. 2	3.7.35	7	8	12	WD	+
	12.12.35	8	T	13	F, C, WD	+
	9.3.36	8·5	30	no second inoculation 19·6	F, P	+
Diarrhœa in May, 1937	4.5.36	8	12	14	F	+
	14.8.36	12	16	20	WD	+
Bacilli in fæces, 8.9.36	19.11.36	7	8	12	P, F, WD	+
	23.4.37	8	10·5	13	P, WD	+
No. 3	3.7.35	7	8	13	F, WD	+
	12.12.35	9	T	10	S, F	—
No diarrhœa	9.3.36	9	12	11	S, F	—
	4.5.36	9	10	12	S, F, WD	—
Bacilli in fæces, 2.11.36	14.8.36	8	11	17·5	W, not P	+
	19.11.36	9	15	not done	P, WD	+
	23.4.37	8	10·5	13	S, Fl, P	+
	20.1.38	8	14	20	F, WD	+
	29.11.38	8	15	20	F, P	+
No. 4	3.7.35	7	13	17	WD	+
	12.12.35	9	S	15	F, C, WD	+
	9.3.36	10	17	19	F, C	+
	4.5.36	8	15	15·5	F	+
Bacilli in fæces, 2.11.36	14.8.36	10	20	no inoculation, 21	F, C, WD	+
	19.11.36	9	12	15	P	+
Diarrhœa in Apr., 1937	23.4.37	9	18·5	not done	WD	+
	20.1.38	10	16	23	F, WD	+
	29.11.38	8	15	20	F, P	+
No. 5	3.7.35	5	14	23	F, WD	+
	12.12.35	7	S, P	23	C, F, WD	+
Bacilli in fæces, 10.3.36	9.3.36	7	8	9	T	—
Diarrhœa in Apr., 1936	4.5.36	6	7·5	7	No T	—
No. 6	3.7.35	5	9	14	F	+
	21.12.35	7·5	S, F	22	F, C, WD	+
	9.3.36	6	14	22·5	F, W, P	+
Diarrhœa in May, 1936	4.5.36	6·5	47	36·5	F, P	+
Bacilli in fæces, 21.6.36	14.8.36	8	12	19	W, slightly P	+
	19.11.36	7	15	not done	D, P	+

Key :—S—small ; F—firm ; C—cool ; P—painful ; WD—well defined ; T—trace ; W—warm ; Fl—flat.

the normal animals it is difficult to avoid the conclusion that an increase of the skin measurements beyond 4 mm. indicated a reaction in an infected animal. It will be seen that two animals, Nos. 1 and 5, both of which were suffering from the acute form of the disease 16 months after infection, gave what were regarded as negative reactions before they were slaughtered although both animals had reacted positively at former tests. No. 1 gave a negative reaction shortly before she became a clinical case and bacilli were found in the fæces, but following the negative test she reacted positively. No. 5 gave two consecutive negative reactions, one shortly before and the other soon after she became a clinical case and was found to be an excretor of bacilli. As an explanation of the failure of these animals to react it might be suggested that a heavy infection had caused a specific desensitisation to those products of the Johne bacillus in the johnin and to which the infected cattle were sensitive. If this be the explanation the desensitisation was transient in one case, for

Table III.

Number.	*Measurements.*			*Remarks.*	*Results.*
	0 Hours.	*48 Hours.*	*72 Hours.*		
1	7	8	7·5		—
2	9	9·5	9·5		—
3	6	7	7		—
4	8	9	10		—
5	7	8	8		—
6	9	12	12		—
7	8	9	9		—
8	8	10	15	firm, cool	+
9	6·5	7·5	8·5		—
10	6	7·5	8		—

the animal became sensitive again while still very heavily infected. But the failure of No. 3 to react at the tests on December 12th, 1935, March 9th, 1936, and on May 4th, 1936, cannot be attributed to the same cause as bacteria were not found in the animal's fæces until several months later, and the absence of any suspicious clinical symptoms until a year after the last test had been made together with the fact that definite symptoms did not appear until more than two years after that test would appear to preclude desensitisation due to a heavy infestation as a cause of the failure. A non-specific desensitisation owing to mere poverty of condition is also excluded. The animal was no doubt infected when the negative reactions occurred and, furthermore, she had previously reacted to the test. Although the johnin used in the test on the cattle was all obtained from the same source there may have been differences in the potency of the different batches, but possible differences in johnin is a very unlikely cause of the discrepancies noted as on the dates these occurred some of the animals showed well-marked reactions.

Discussion.

Outbreaks of Johne's disease in sheep have been recorded by a number of workers including Stockman (1911), Lange and Berge (1915), McFadyean and Sheather (1916), Howarth (1932), Johnstone (1934) and Thiery and Getas (1935). The evidence, however, does not suggest that the disease is common among flocks and it is most improbable that its incidence is as high as that of the disease in cattle. However, the fact must not be overlooked that the symptoms in sheep are less likely to arouse suspicion than they are in cattle and, furthermore, fewer sheep carcases will be opened and even cursorily examined than cattle carcases, which all tends to increase the chances of the disease in sheep being overlooked. The movements of individual sheep from flock to flock are more limited than the movements of individual cattle from herd to herd and on this account there is probably less danger of dissemination of infection among flocks than among herds. Johne's disease in sheep, which may take an annual toll of 5 per cent. or more, cannot be regarded as a pathological curiosity, whereas tuberculosis of sheep is so uncommon and affects so few individuals that it may almost be classed as such. In cattle, tuberculosis is much the commoner disease. It might, therefore, be concluded that sheep are relatively resistant to tuberculosis and susceptible to Johne's disease by virture of inherent qualities, or that sheep husbandry is much more favourable to the spread of Johne's disease than it is to the spread of tuberculosis. A general discussion on the dissemination of the two infections is not appropriate but it should be emphasised that with both species of animals the clinical manifestations of Johne's disease are more prone to occur soon after parturition and that in both species dissemination will be most abundant at this time. Ewes lamb in flocks and more or less simultaneously and the lambs form part of the flock. Cows calve more or less throughout the year and their young rarely run or mix with the herd but are segregated soon after or immediately after birth, generally even from their own dams. Tuberculosis infection has little respect for age and, therefore, a high incidence of the disease is found in adult cattle that are housed. Johne's disease, however, does not reach such a high incidence and this may be due to the fact that the adult is resistant to infection and that the young by being segregated from infected animals are not exposed to heavy risks. On the other hand, the freedom from tuberculosis enjoyed by sheep may be attributed to their never being housed, whereas the comparatively heavy losses Johne's disease has occasionally been shown to cause may be attributed to the exposure of the young to infection.

The possibility of sheep becoming infected from cattle has been recognised by most workers but a direct history of affected sheep having been associated with cattle is not always found. In those cases, where the bacilli from sheep have been readily cultivated it is probable that it was the same micro-organism that caused the disease in cattle even although there may have been little or no direct or immediate

contact of the two species of animals. Thus McFadyean and Sheather (1916) cultivated the Johne bacillus from sheep on a farm where very few cattle were kept and where Johne's disease was not known to have occurred among the cattle; and Howarth (1932) had apparently no difficulty in cultivating the micro-organism from sheep in California that never came into contact with cattle. Twort and Ingram (1913) cultivated the bacillus from a sheep but did not state the animal's history. That there may be differences in the bovine and ovine strains of the bacillus is suggested by Dunkin and Balfour-Jones' (1935) report on the strain they isolated from sheep, that grew more slowly than a bovine strain, only a few colonies being just apparent to the naked eye after seven months' incubation and on only three out of twelve tubes inoculated.

The inability in the present instance to cultivate the bacillus further strengthens the probability of there being two strains of the bacillus, the less readily cultivated of the two being a cause, but not the invariable cause, of disease in sheep, and the successful experimental infection of six calves shows that this strain is a potential source of infection for bovines. The failure to set up infection in adult sheep indicates a definite age resistance to infection similar to that which probably exists in cattle. Meyer (1913) drew attention to the susceptibility of calves and the fact that successful transmission experiments had all been done on young animals, and Hagan (1938) records the successful infection of animals up to one year of age, but he failed to infect three animals exposed to infection when over two years old. Up to the present, however, no very systematic experiments have been made on the relative resistance of animals at different ages. If as appears probable susceptibility were shown to decrease with age definite information on this point might be of value in framing and influencing methods of animal husbandry directed towards the control and prevention of the disease. The close parallel between the disease in sheep and cattle indicates that the sheep might be a useful experimental animal on which to make further experimental studies.

The intradermal inoculation of the six experimentally-infected cattle with johnin gave irregular results that are, however, in general accord with similar tests carried out on naturally infected cattle. Thus Broerman and Fogle (1932) found twelve positive reactors and one doubtful reactor at *post-mortem* examination, whereas no disease was found in seven animals that reacted to the test.

Furthermore, three cattle that had the disease did not react. Houthuis (1932) tested 44 cattle purchased apparently because they were regarded as suspicious cases and tested a number of the animals with two different johnins. Of 41 tested with one johnin 27 reacted and 11 failed to react; with the other johnin 25 out of 28 reacted and three failed to react. In both cases, all of these animals were found infected at *post-mortem* examination. It should be noted that 42 of the 44 animals selected on clinical grounds, assisted probably

in some cases by an examination of the fæces, were found infected when slaughtered. The diagnosis without the aid of johnin, therefore, gave more consistent results than would have been the case had reliance been placed on johnin alone. Minett (1935) carried out *post-mortem* examinations on 48 cattle that had reacted to the test; 39 of these were found to be infected but in seven the disease was not diagnosed. Seven other animals reacted and on clinical grounds only were diagnosed as cases of Johne's disease. Six animals which were clinical or suspected cases of Johne's disease were examined *post mortem* and found infected but none had reacted to the test. Hagan and Zeissig (1933) found the intradermal johnin test less reliable than the test in which a positive reaction depends upon a rise of body temperature following the intravenous inoculation of either johnin or avian tuberculin. More encouraging results were, however, reported earlier by Dunkin (1928) who tested 46 to 48 animals that were known to be affected with Johne's disease. Details of these tests are not given, but all the animals reacted except those that were very advanced cases. Before the johnin was used in the field 18 farm cattle were tested and two reacted. It may be concluded that the intradermal tests gives an undesirable margin of error which has so far not been eliminated. The present series of tests on cattle and also on sheep, even bearing in view the fact that the cattle were infected with an ovine or at least an atypical strain of the Johne bacillus as far as cultural requirements are concerned, underlines the desirability for further study of the allergic state in Johne's disease.

Summary.

An outbreak of Johne's disease in a flock of sheep is recorded and the symptoms and lesions of the affected sheep are described. The causal micro-organism resisted cultivation and in so far it differed from the Johne bacillus isolated from cattle.

The infection was readily transmitted to lambs but adult sheep were resistant.

Six calves were exposed to heavy infection and all became infected.

Johnin diagnostic tests were carried out on sheep and on the experimental cattle. The results of these tests were unsatisfactory.

I wish to record my appreciation and thanks to the Agricultural Research Council for a grant that enabled the infection experiments to be carried out. I also record my thanks to Dr. F. C. Minett for kindly supplying the johnin that was used in the testing of the sheep and my thanks to Mr. G. W. Dunkin for kindly supplying the johnin used for the tests on the experimental cattle.

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